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EPIDEMIOLOGY AND RECENT EPIDEMICS.*

By SIMON FLEXNER, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Since the last congress, held in 1916 in the midst of the racking uncertainties of the great war, notable events and serious calamities have befallen the world and arrested the attention of all thoughtful men. A bitter and passionate military contest has been brought to a hopeful conclusion; but because of the unparalleled cost of the struggle, in lives and in treasure, deep unrest, revolution even, starvation and disease are prevailing over a large part of Europe, while also within the three year period elapsed between the last congress and the present one three destructive epidemics of disease have ravaged the United States and the world.

Hence it has seemed fitting to me that on this occasion and before this representative body of medical men we should pause for a brief period in order to review, as it were, our knowledge of epidemics and at the same time of the practical hygienic measures, based on this knowledge, which we have already put or in ordinary course of events may reasonably hope to put into motion against the spread of these epidemics, so that we may form a judgment of the efficacy of such measures and arrive possibly at new points of view from which to launch a more decisive attack. Moreover, it seems imperative that we should consider not only the sum of our knowledge of epidemics, but also certain facts relating to the populations affected which react powerfully on the successful application of the hygienic means available.

Three epidemic diseases—poliomyelitis, streptococcus pneumonia and influenza—have been especially destructive in the western world during the past three years; also, because of certain common characteristics, they are well adapted for the purpose I have in view.

* President's address, read before the Tenth Congress of American Physicians and Surgeons, Atlantic City, N. J., June 16, 1919.

Poliomyelitis.

In the United States we are becoming increasingly familiar with epidemics of poliomyelitis. Prior to 1907, infantile paralysis was a rare disease in this country; since then it has prevailed fitfully every summer and autumn, and in one notable instance, at least, in the winter season,[1] claiming victims by the score or hundred, until in 1916 an outbreak of unprecedented severity, with its center of violence in New York State, swept over a considerable number of states.

Fundamental knowledge of poliomyelitis may be said to have grown rapidly since Wickman's epochal clinical studies published in 1907. We are, indeed, today in possession of precise information covering essential data with regard to the nature of the inciting micro-organism, notwithstanding its very minute size, and also concerning the manner in which it leaves the infected or contaminated body within the secretions of the nasopharynx chiefly and gains access to another human being by means of the corresponding mucous membranes and apparently in no other way. Moreover, the inciting virus so called, up to the present time and notwithstanding many and assiduous efforts, has not been detected apart from the infected or merely contaminated human being, and there is therefore no foundation in ascertained fact for an assumption that the virus is conveyed to persons otherwise than by other persons who harbor it.

Streptococcus Pneumonia.

The second example, namely, that of streptococcus pneumonia, presents a phenomenon almost, if not quite, new among the epidemic diseases. It appears as if during the winter of 1917–1918 there occurred in several localities within the United States, and also, but in less degree, in France, at least a great increase in the incidence of a type of pneumonia which previously had been very infrequent. It appears also that the greatest number of cases and of fatalities arose in the United States in the military cantonments; that the disease first prevailed as a secondary pneumonia following measles; but that before long the severity of the infection was such that cases

1. At Fairmount, W. Va.

of primary streptococcus pneumonia began to arise. Moreover, at this juncture the disease spread from the military to the civil populations.

The nature of the micro-organism inducing this form of epidemic pneumonia is indicated in the name which the disease has come to bear. The difficulty in this instance has not been in finding out the inciting microbe, but rather in differentiating the streptococci responsible for the epidemic disease from streptococci possessing the ordinary pathogenic properties, or even from those of saprophytic nature so commonly present on the upper respiratory mucous membranes without provoking widespread disease. However, numerous studies of the bacteriology of this epidemic of pneumonia, at distinct and often widely remote cantonments, showed that the microbic incitant was in almost every instance *Streptococcus hemolyticus*. Moreover, because of the wide occurrence of the epidemic pneumonia, this type of streptococcus could be found in normal throats and as a secondary invading micro-organism in the lungs in cases of ordinary lobar pneumonia. Thus far very little progress has been made in the classification of streptococci, which form a class apparently even more heterogeneous than the pneumococci.

The point I wish to emphasize is this: Regarding epidemic diseases in general we are wont to assume the introduction from without, and usually from a distant locality, of a special kind or race of micro-organism which is held directly responsible for the epidemic ensuing. In the instance of the epidemic pneumonia, no such importation or new introduction of the inciting streptococcus needs to be or actually is assumed. It is so probable as to amount to practical certainty that the excessively virulent *Streptococcus hemolyticus* was developed by a process of selection, through successive transfer from person to person, and by gradual enhancement of its invasive properties.

We are in this case on safe grounds when we assert that the inciting streptococcus is always personally borne: that is, that it leaves the respiratory organs of one person to be introduced on the corresponding organs of other persons, and in no other manner. In other words, the mechanism or mode of infection in epidemic streptococcus pneumonia, as in poliomyelitis, may be said to be clearly apprehended.

Influenza.

The case of influenza, the third and perhaps the most important example cited, is quite different, since wide divergences of belief and opinion regarding the nature of the inciting micro-organism and the manner of infection still prevail. The reasons for these differences are several, but the most important, perhaps, relates to the common observation of the manner of spread or attack of the disease. To the casual observer there is something uncanny in the way influenza strikes down its victims. While other epidemics proceed from bad to worse, with at least progressive increases of intensity, influenza seems to overwhelm communities over even wide stretches of territory as by a single, stupendous blow. While in the one case the gradually accelerating rate of speed of extension may be taken to indicate personal conveyance of the provoking micro-organism, in the other the sudden wide onset appears the very negation of personal communication.

Hence the invoking of mysterious influences, the revival of the notion of miasm and similar agencies, to account for this phenomenon. Indeed, the public mind in general lends itself readily to such formless concepts, for the reason that there still resides in the mass of people, even in the more enlightened countries, a large uneradicated residue of superstition regarding disease. One does not need to look far or dig deep in order to uncover the source of this superstition. We have only recently emerged from a past in which knowledge of the origin of disease was scant, and such views as were commonly held and exploited were mostly fallacious. It is, indeed, very recently, if the transformation can be said to be perfect even now, that the medical profession as a whole has been completely emancipated. All this is very far from being a matter of remote importance only, since in the end the successful imposition of sanitary regulations involves wide cooperation; and until the majority of individuals composing a community is brought to a fair level of understanding of and belief in the measures proposed, serious and sustained endeavor to enforce them is scarcely to be expected.

And yet no better instance of a communicable disease could perhaps be invoked than influenza to exorcise the false idea of the

mysterious origin of epidemics. To dwell solely on the sudden and overwhelming stroke of the disease is wholly to overlook the significant incidents that precede the mass infection, because they are of such ordinary nature and lack all dramatic quality. Accurate observers noted long ago that influenza in its epidemic form did not constitute an exception to the common rule governing epidemic diseases, which were obviously associated with persons and their migrations. What the early students made out by tracing the epidemic backward to its point of departure, more modern observers have confirmed by carefully kept records, often graphically compiled, as in the excellent instance of the Munich records covering the epidemic of 1889–1892, which can now be supplemented by a number of similarly constructed records of the epidemic just passed. These detailed records show convincingly a period of invasion during which there is a gradual rise in the number of cases, to culminate, within a period variously estimated at from one to three weeks, in a widespread, so-called "explosive" outbreak of the disease.

It happens that the early cases of epidemic influenza tend not to be severe, chiefly because they rarely are attended by pneumonia and hence are frequently mistaken, and the confusion in diagnosis is resolved only when the full intensity of the epidemic is realized. In the meantime, rich opportunity has been afforded for the free and unrestricted commingling of the sick and the well, of doubtless healthy carriers of the inciting agent and others, until so high a degree of dissemination of the provoking micro-organism has been secured as to expose the entire susceptible element of the population, which happens to be large, to an almost simultaneous response to the effects of the infecting microbe.

Deductions of like import can be drawn from the geographic movements of influenza epidemics. In eastern Russia and Turkestan, influenza spreads with the pace of a caravan, in Europe and America with the speed of an express train, and in the world at large with the rapidity of an ocean liner; and if one project forward the outcome of the means of intercommunication of the near future, we may predict that the next pandemic, should one arise, will extend with the swiftness of the airship. Moreover, not only is this rate of spread determined by the nature of the transportation facilities of the region

or the era, but towns and villages, mainland and island, are invaded early or late or preserved entirely from attack according as they lie within or without the avenues of approach or are protected by inaccessibility, as in instances of remote mountain settlements and of islands distant from the ocean lanes or frozen in during winter periods.

It is desirable, in the interest of clear thinking, to carry this consideration of the characteristics of epidemic influenza a step farther. A feature of the epidemic disease of particular significance is the tendency to recur, that is, to return to a stricken region after an interval, usually of months, of relative quiescence. Thus the beginnings of the last pandemic in western Europe and the United States have been traced to sporadic cases appearing in April, May and June, possibly even earlier in certain places, while the destructive epidemic raged during September, October and November of 1918. There are very good reasons for believing that in itself influenza is not a serious disease, but that its sinister character is given by the remarkable frequency with which it is followed, under particular circumstances, by a concomitant or secondary pneumonic infection to which the severe effects and high mortality are traceable. Now it is this high incidence of pneumonia, the product of invasion of the respiratory organs with bacteria commonly present on the upper respiratory mucous membranes—streptococci, pneumococci, staphylococci, Pfeiffer's bacilli and even meningococci—that stamps the recurrent waves of the epidemic with its bad name.

If we compare the pneumonic complications of influenza with those which arose in the cantonments in 1917–1918, first as attendants of measles and later as an independent infection, we note immediately that in both instances the severe effects and high fatalities arose, not from bacteria brought or imposed from without, but from their representatives which are commonly resident on the membranes of the nose and throat in health. Whatever we may have to learn of the micro-organism inducing measles, still undiscovered, and of influenza, still under dispute, and their mode of invasion of the body, no one would question that the bacteria inducing pneumonia are personally borne.

Efficacy of Public Health Measures.

With these various considerations before us, we may now discuss the question of the efficiency of our public health measures in diminishing the incidence of epidemic diseases. It should now be evident that our three examples are essentially instances of respiratory infections, that is, diseases in which the inciting micro-organism enters the body by way of the air passages, although not necessarily, as in poliomyelitis, directly injuring those parts. Protection in diseases of this class is not to be secured by applying sanitary measures on a wide scale to an extraneous and inanimate source of the infectious micro-organisms, as to water supplies contaminated with the dejecta of typhoid patients, or even to inferior animal species, such as the mosquito or the rat, which act as intermediaries in conveying the germs of yellow fever or of infectious jaundice; but it is alone to be attained by methods of personal hygiene, applied on the individual scale of safeguarding one person from another, the most difficult of all hygienic regulations to enforce.

Returning now to epidemic poliomyelitis, we may fairly claim that we are in possession of the essential facts which, if widely applicable, should enable us to control the spread of that disease. But we can, I think, hardly claim that up to the present time our accomplishments in that direction have been remarkable. It is sufficient merely to compare the curve of incidence of the Swedish epidemic of 1905, before the nature and mode of infection of poliomyelitis were known, with those of the last several years, in Massachusetts and New York State for example, in order to conclude that the progress of the epidemics in the several places was practically identical.

And, indeed, this is what might be expected in view of the difficulties surrounding the prompt and accurate diagnosis of poliomyelitis in its atypical and abortive, often ambulant forms. Once the disease is introduced under conditions favoring its epidemic spread, a wide dissemination of the inciting micro-organism takes place, and a constantly increasing number of persons becomes exposed to its presence before any restrictive measures are put into effect, and indeed also after they have been applied. In the case of poliomyelitis, as in that of influenza itself, a wide distribution of the infectious

agent precedes the enforcement of preventive sanitary regulations. These considerations do not, of course, warrant intermission of the protective measures now in use, which undoubtedly save many individuals from exposure and thus form potential attack; they do, however, offer an explanation of why, up to the present time, greater success has not attended efforts at control once the epidemic is under full way.

The case with the epidemic pneumonias is of another order: They represent theoretically two diseases which should respond to methods of control based on our knowledge of their mode of infection. In the epidemic streptococcus pneumonia and the pneumonia following influenza we are dealing with pathologic conditions in which not a newly introduced, extraneous micro-organism is operating widely and insidiously, but one in which the active microbes concerned are examples merely of intensified races of common and almost omnipresent species belonging to the flora of the nasopharynx. The infectious agents in these instances are contained within the nasal, buccal and bronchial secretions, and are disseminated in the sprayed material which is coughed or otherwise thrown into the surroundings of the patients. The lesson, therefore, to be derived from the severe experience of the recent pneumonia epidemics is to the effect that measles and influenza patients are not to be assembled into large groups or kept in open wards, but should be placed in separate rooms or cubicles, where they and their attendants may be preserved as far as possible from sputum droplet contamination. In the instance of epidemic pneumonia, a chain of direct infection from one patient to another tends to be established, and hence the sanitary control of those diseases is to be sought through the breaking, as it were, of this vicious circle.

A distinction has now been intimated in the possibilities of direct sanitary control between the two epidemic diseases—namely, poliomyelitis and influenza—introduced from without, and the pneumonias, which are mere, if intense, exaggerations of sporadic diseases ordinarily prevailing. I propose now to lay before you a suggestion as to means of attacking the exotic epidemic diseases which may come to merit serious attention.

Eradication of Diseases in Endemic Foci.

Epidemic diseases in the commonly accepted sense have fixed locations—the so-called endemic homes of the diseases. In those homes they survive without usually attracting special attention often over long periods of time. But from time to time, and for reasons not entirely clear, these dormant foci of the epidemics take on an unwonted activity, the evidence of which is the more frequent appearance of cases of the particular disease among the native population, and sooner or later an extension of the disease beyond its endemic confines. Thus there are excellent reasons for believing that an endemic focus of poliomyelitis has been established in northwestern Europe from which the recent epidemic waves have emanated.

Similarly, there are excellent reasons for regarding the endemic home of influenza to be eastern Europe, and in particular the border region between Russia and Turkestan. Many recorded epidemics have been shown more or less clearly to emanate from that area, while the epidemics of recent history have been traced there with a high degree of conclusiveness. From this eastern home, at intervals usually of two or three decades, a migrating epidemic influenza begins, moving eastward and westward, with the greater velocity in the latter direction.

Now since the combating of these two epidemic diseases, when they become widely and severely pandemic, is attended with such very great difficulty and is of such dubious success, and this notwithstanding the prodigious public health contests which are waged against them in which the advantages are all in favor of the invading micro-organismal hosts, it would seem as if an effort of central rather than peripheral control might be worth discussion. According to this proposal, an effort at control amounting even to eventual eradication of the diseases in the regions of their endemic survival would be undertaken, an effort, indeed, not occasional and intensively spasmodic, as during the pandemic excursions, but continuous over relatively long periods, in the hope that the seed beds, as it were, of the diseases might be destroyed.

That such an effort at the eradication of a serious epidemic disease may be carried through successfully the experience with yellow

fever abundantly proves. In attacking that disease, the combat was not put off until its epidemic spread had begun and until new territory, such as New Orleans, Jacksonville and Memphis, had been invaded; but the attack was made on its sources at Havana, Panama and now Guayaquil, to which endemic points the extension into new and neutral territory had been traced.

I do not disregard the essential fact, in bringing this suggestion forward, that the control at its sources of yellow fever is quite another and probably far simpler problem than the control in their endemic foci of poliomyelitis and influenza. It is, perhaps, unnecessary to go far into the reasons why the latter would doubtless prove to be far more difficult of accomplishment than has been the former. I am not now engaged in presenting a plan of operation or proposing that the attempt at eradication be made immediately. Our knowledge of all the facts involved in the epidemiology of poliomyelitis, and especially of influenza, may still be too imperfect for immediately effective action. But the very magnitude of the problem of these otherwise uncontrollable epidemic diseases invites to an imaginative outlook which, while perhaps nonrealizable today, may not, in view of the rapidly advancing knowledge of the infectious diseases, be hopelessly out of reach tomorrow.

Nor am I insensible to the labor and cost in money and talent which the setting out on such an ambitious enterprise would entail. But here, at least, is a world problem of such proportions and nature as to invite the participation of all the scientifically advanced countries in a common effort to suppress one of the most menacing enemies of civilized man and of human progress.

In proposing to strive for the high achievement, not merely of parrying the blows struck by destructive epidemics, but of rendering them impotent to strike in the future, we may pause for a moment to reflect on the different ways in which peoples react to great calamities, such as those brought by war and by disease. As the results of a cruel and devastating war, revolutions in governments supposed the most stable may occur; no such result follows on still more devastating epidemics. The recent epidemic of influenza claimed, possibly, more victims than did the great war, and the losses to the world in emotion spent, treasure consumed, and progress impeded are

incalculable; yet, through a fortuitous circumstance of psychology, from the one calamity the world may emerge chastened, perhaps even bettered, while from the other, because of a depth of ignorance amounting often even to fatalism, mankind may largely miss the deep meaning of the lesson.

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ETIOLOGY OF YELLOW FEVER.

IX. Mosquitoes in Relation to Yellow Fever.

By HIDEYO NOGUCHI, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, July 3, 1919.)

That a certain species of mosquito serves as a carrier of yellow fever has long been suspected. Finlay[1] supported this theory as early as 1881 and went so far as to advise conferring immunity upon non-immune persons by causing them to be bitten by mosquitoes (*Stegomyia calopus*) which had previously fed on a yellow fever patient. Thenceforth *Stegomyia calopus* became the center of attention in yellow fever investigations, the theory being experimentally confirmed finally by Reed, Carroll, Agramonte, and Lazear,[2] who successfully transmitted yellow fever to non-immune human subjects by allowing the infected stegomyias to bite them. The successful eradication of yellow fever by Gorgas[3] from various endemic centers in the western hemisphere through anti-stegomyia campaigns amply attests the correctness of this important discovery. Reed and his coworkers established the fact that the virus exists in the peripheral blood during the first 3 days of the illness and that the mosquitoes, which have fed on a yellow fever patient during this period, become capable, after about 10 days, of transmitting the infection by their bite to a normal person. Hence they assumed that the virus required a period of at least 10 to 12 days of extrinsic incubation during which it must pass through a life cycle, as was known to be the case with the parasite of malaria. The necessity for an extrinsic incubation of about 12 days was pointed out by Carter[4] in his epidemiological studies of yellow fever. This theory, coupled with the improbability of a bacterial origin of the disease, brought about the general impression that the virus was a protozoan, although nothing definitely objective was adduced in support of this view.

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[1] Finlay, C., El mosquito hipotéticamente considerado como agente de trasmisión de la fiebre amarilla, *An. r. Acad. cien. méd. Habana*, 1881–82, xviii, 147.

[2] Reed, W., Carroll, J., Agramonte, A., and Lazear, J. W., The etiology of yellow fever, *Am. Pub. Health Assn., 28th Meeting*, 1900; also *Senate Doc. No. 822, 61st Congr., 3rd Sess.*, 1911, 56.

[3] Gorgas, W. C., Sanitation in Panama, New York and London, 1915.

[4] Carter, H. R., Note on the spread of yellow fever in houses. Extrinsic incubation, *Med. Rec.*, 1901, lix, 933.

Reference has already been made[5,6] to the fact that the virus of yellow fever passed through the pores of a Berkefeld or a Chamberland filter. This point is important in excluding as the inciting agent of the disease any organism incapable of passing through these filters which might be found incidentally in the blood or tissues of yellow fever patients. Some persons believe that the virus is ultramicroscopic, at least with the magnification possible at the present time.

The characteristics of the organism isolated from the yellow fever cases in the present investigation conformed with all the other known characteristics of the yellow fever virus, and it became necessary to determine whether or not it behaved like the latter in relation to mosquito transmission. That it does, under certain conditions, is shown in the experiments to be described.

Material and Mode of Experiments.

Larvæ of *Stegomyia calopus* collected from houses in Guayaquil were brought to the laboratory.[7] They varied in stage from the very young to the pupal form.

For the purpose of maintaining the imagos which emerged from these larvæ special cages were constructed consisting of a square wooden frame with wire net on all but two opposing lateral walls, which were solid pieces of wood with a round opening in the center for the insertion of the arm or a glass of water from either side of the cage. The opening was covered by a well fitting sliding door which could be drawn to one side and slipped back in place. A long glass cylinder about 8 inches long and 2 inches in diameter with one end closed was used for handling individual mosquitoes. When the specimens were to be killed for examination a piece of cotton saturated with chloroform was put at the bottom of the cylinder. For histological study the mosquitoes were placed in fixing fluid such as sub-

[5] Reed, W., and Carroll, J., The etiology of yellow fever; a supplemental note, *Senate Doc. No. 822, 61st Congr., 3rd Sess.*, 1911, 149.

[6] Marchoux, Salimbeni, and Simond, La fièvre jaune, *Ann. Inst. Pasteur*, 1903, xvii, 665.

[7] These were obtained through the cooperation of the officials of the Municipal Board of Health of Guayaquil.

limate-alcohol, Zenker's fluid, or 10 per cent formaldehyde, after the wings and legs were trimmed off. Mosquitoes engorged with blood usually sink to the bottom in a short time, but those which are unfed or have already digested the blood do not sink for many hours or even days, and it was necessary to keep them within the fixing fluid by forcing them down with a piece of absorbent cotton. In some instances a minute needle puncture into the abdomen or thorax, or both, facilitated the prompt penetration of the fixative agents. The specimens fixed in sublimate-alcohol or Zenker's fluid were washed in water for 48 hours and then transferred to 50 per cent alcohol, in which they were kept until worked out later for serial sections.

The larvæ showed a surprising resistance to phenol (3 per cent), formaldehyde (10 per cent), sublimate-alcohol (two parts of saturated aqueous solution of mercuric chloride and one part of absolute alcohol), and Zenker's fluid, being capable of surviving for at least 15 minutes. Some lived as long as 30 minutes in 10 per cent formaldehyde.

Monkeys and infected as well as normal guinea pigs, when not allowed to defend themselves, are eagerly bitten by the stegomyias. Rats, however, are seldom bitten by these mosquitoes, and bats have never been seen to be attacked when placed in a cage with hungry stegomyia females.

Transmission Experiments with Stegomyia calopus from Man to Animals.

Attempts were made to reproduce the appearances of yellow fever in the guinea pig by permitting the stegomyias to bite the animals after having fed upon the blood of yellow fever patients during the early stage of the disease. The procedure consisted in putting one arm of the patient into a cage containing 100 to 300 mosquitoes which had been hatched from the larvæ in captivity, and allowing the mosquitoes to feed until most of the females were engorged with blood. The cage containing the insects was then carefully kept in the shadow; sometimes the females were transferred to a separate cage. A glass of water containing a pea and a green twig was put into the cage, and each morning a slice of banana or papaya was placed upon the wire wall.

Since an enormous amount of labor would be required to test the infectivity of each female mosquito, summary experiments were made; that is, a dozen or more mosquitoes were allowed to bite one and sometimes two animals at the same time. The animals were then placed in separate cages and kept under daily observation for a period of at least 1 month, in case no infection took place within a shorter time. The tests for infectivity were usually made 2 weeks after the feeding on the patients, but sometimes earlier.

As the protocols show, positive leptospiral transmission was obtained in one of the following experiments.

Experiment 1 (*Negative*).—Aug. 5, 1918. 40 recently hatched females of *Stegomyia calopus* were allowed to become engorged by feeding on the right arm of Case 9 (G. C.), a severe case of yellow fever, admitted on the 3rd day of disease to the hospital. The patient recovered. Aug. 10. Sixteen females surviving. Eggs were found on the wet leaves in the glass. The sixteen mosquitoes surviving were allowed to feed on a very young normal guinea pig on that day; that is, 5 days after feeding on the patient's blood. All became engorged within the period from 12 m. to 2 p.m. The guinea pig was removed for observation into another cage. It remained well with normal temperature (38–39.5°C.) for 11 days, when it died of an intercurrent disease. There was no jaundice or change in the liver or kidney.

Experiment 2 (*Positive Transmission*).—Aug. 15. Twelve of the sixteen mosquitoes used in the above experiments still surviving. Larvæ in the glass. Aug. 28. Eight still surviving (23 days after feeding on the patient). A normal guinea pig was placed in the cage with these eight mosquitoes, which immediately attacked the animal, all becoming engorged. The guinea pig was removed to another cage and kept under observation. Temperature 38°C. Aug. 29, temperature 38°C.; Aug. 30, 40°; Aug. 31, 38.6°; Sept. 1, 39.3°; Sept. 2, 38°; Sept. 3, 37.5°; Sept. 4, 36°. Death occurred in 6 days.

Autopsy.—Moderately marked general jaundice. The lungs showed several small ecchymoses; the liver was yellowish brown and mottled; the kidneys were congested and showed ecchymoses; the stomach contained blood-stained food; the intestines showed numerous serous and mucous ecchymoses, and the stool was mixed with blood; the bladder was half full of yellowish brown urine which contained an abundance of albumin and casts; the adrenals were congested; the spleen, pancreas, and testes were apparently unchanged; the heart showed a few minute ecchymoses on the surface.

Microscopic Examination.—Examinations of emulsions of the liver, kidney, and blood for the leptospira were negative. The infectivity of the liver and kidney was tested on another guinea pig by injecting organ emulsions into the subcutaneous tissue. This animal died under the same symptoms in 7 days.

The leptospira was demonstrated in the kidney in small numbers, but not in the liver or blood.

Experiment 2 shows that the mosquitoes which sucked the blood of a yellow fever patient on the 3rd day of the disease were infective to the guinea pig after a period of 23 days. That they were unable to produce a typical infection when tested 5 days after the feeding is shown in Experiment 1.

Experiment 3 (*Negative*).—Aug. 5, 1918. Twenty-eight females were allowed to become engorged on the right arm of Case 22 (A. M.), a fatal yellow fever case, admitted to the hospital on the 4th or 5th day of the disease. The patient died on the 10th day with typical symptoms. Aug. 10. Twelve surviving. Aug. 12 (7 days after feeding). The mosquitoes were placed on a normal guinea pig. Several became engorged, and several were killed by the animal. This guinea pig showed no symptoms within the period of observation and was discarded.

Aug. 28. The surviving females were allowed to bite a normal guinea pig. The temperature rose to 39.9°C. on the 5th day but soon returned to normal and the animal showed no symptoms afterwards.

Experiment 4 (*Doubtful*).—Aug. 14, 1918, 4.15 p.m. A large number of stegomyias were placed on the left arm of Case 10 (M. N.), admitted on the 2nd day of the disease. 60 females which had become fully engorged were put into another cage. The patient died of typical yellow fever on the 7th day. Aug. 15, 9.30 a.m. Another feeding by the same mosquitoes on the same patient. Aug. 20. 34 surviving. Aug. 27 (13 days after feeding). 29 surviving. These were allowed to bite a normal guinea pig, and all became engorged. The guinea pig showed the following symptoms: Aug. 28, temperature 37.6°C.; Aug. 29, 38°; Aug. 30, 38.2°; Aug. 31, 39.5°; Sept. 1, 39.8°; Sept. 2, 40°; Sept. 3, 38.4°; Sept. 4, 38.1°; Sept. 5, 37.8°; Sept. 6, 38°; Sept. 7, 38.2°; Sept. 8, 38.1°. There was a trace of icterus in the scleras on the 8th and 9th days, which faded during the following day.

As the animal returned quickly to normal without further symptoms, a positive diagnosis was not possible, but it is probable that there was a mild infection.

Experiment 5 (*Negative*).—Aug. 14, 1918, 5 p.m. The right arm of Case 8 (R. V.), admitted on the 2nd day of disease, was put into a cage containing about 50 mosquitoes. Only five females sucked the blood within about 30 minutes. The case was severe but recovered. Aug. 15, 12 m. The feeding was repeated, 32 females becoming engorged immediately. Aug. 25. Many minute larvæ were found in the vessel containing water. Aug. 28, 10 a.m. The surviving females, thirteen in number, were allowed to feed on a normal guinea pig. All eagerly

engorged. The guinea pig was placed in a separate cage for observation. As no symptoms developed within a month it was discarded.

Experiment 6 (Negative).—Aug. 28, 1918, 12 m. 80 female stegomyias were allowed to engorge on Case 52, a girl of 10 years, whose brother had died in the hospital 4 days previously with yellow fever, but who was showing only a suspicion of icterus in the scleras which disappeared on the following day. The case was so mild that the patient never was confined to bed and left the hospital in 7 days. The temperature did not exceed 39°C. and remained at that point for the 1st day only. If the case was one of yellow fever it was extremely mild. Sept. 11. 23 females surviving. These were allowed to feed on a normal guinea pig; all became fully engorged immediately. The guinea pig was put into a separate cage for observation for 10 days. No symptoms developed and the animal was discarded after 3 weeks.

Transmission Experiments with Stegomyia calopus from Animal to Animal.

In earlier experiments[8] on *Leptospira icterohæmorrhagiæ* transmission of the disease from one guinea pig to another by means of *Culex pipiens* was unsuccessful. The present study with *Leptospira icteroides*, however, brought out the fact that *Stegomyia calopus* is capable of transmitting the experimental disease resembling yellow fever from an infected to a normal guinea pig. The positive results are few, notwithstanding the numerous attempts made, but are sufficiently conclusive to establish the main point, that this mosquito may serve as an intermediary host of *Leptospira icteroides*. The term intermediary host, however, is not used here in the sense understood in the case of certain protozoan organisms, which require an extrinsic host in which to pass their life cycle, but denotes that a certain length of time is necessary for multiplication of the organisms to such numbers that the mosquito may transmit enough to produce infection. From the biological and cultural properties of the organism this hypothesis seems reasonable, though the possibility of a stage of development in the mosquito has not been excluded. The following experiments indicate the positive transmission of *Leptospira icteroides* from animal to animal by the bite of the stegomyia mosquito.

[8] Noguchi, H., The survival of *Leptospira* (*Spirochæta*) *icterohæmorrhagiæ* in nature; observations concerning microchemical reactions and intermediary hosts, *J. Exp. Med.*, 1918, xxvii, 609.

Experiment 7 (*Negative*).—Aug. 4, 1918. 50 female stegomyias were engorged with the blood of a guinea pig which had been infected with the Case 1 strain of *Leptospira icteroides* 6 days previously. The animal was showing the organisms in the blood and had all the characteristic symptoms. Aug. 8. The mosquitoes fed on another guinea pig with beginning fever and albuminuria. The blood contained occasional leptospiras. Aug. 10. The mosquitoes which were used later in the experiments recorded below were allowed to bite a normal guinea pig on the 6th day after the first feeding. Only five mosquitoes attacked the animal. No symptoms followed the biting, and the guinea pig was discarded as negative on Sept. 13.

Experiment 8 (*Positive*).—Aug. 16 (12 days after the first and 8 days after the second feeding). Two normal guinea pigs were placed in the cage with the mosquitoes. Guinea Pig 544 was bitten by nineteen mosquitoes. The highest temperature was 39.6°C. on the 5th day, and the animal became mildly icteric on the 8th day. On the 12th day the temperature dropped to 37° and death ensued.

Autopsy.—Fatty degeneration of the liver, recent hemorrhagic spots in the lungs, semidigested bloody contents in the stomach and intestines, acute parenchymatous nephritis, and some ecchymoses in the lymph glands. The icterus was very slight. The leptospira was demonstrated under the dark-field microscope.

Guinea Pig 545 was bitten by nine other mosquitoes on the same day. The highest temperature was reached on the 6th day, but the animal gradually returned to normal without any icterus. It remained well for 24 days and was then subjected to a second infection with the same strain of the organism on Sept. 7. It proved refractory to the infection.

Experiment 9 (*Negative*).—The mosquitoes used in Experiment 8 were kept for another experiment, but within 5 days only eight of the twenty-eight still survived. These were allowed to feed on another normal guinea pig (No. 571) on Aug. 21. The animal showed no symptoms after the biting and was discarded as negative on Sept. 7.

Experiment 10 (*Positive*).—Aug. 13, 1918. In this series four guinea pigs (Case 1 strain) infected at different stages of the disease, 6th, 7th, 8th, and 9th days after the inoculation, all showing the symptoms, some intensely icteric, and some showing the leptospiras in the blood, were used to infect the mosquitoes in four separate cages. Three of the guinea pigs died subsequently, and one recovered. 142 engorged females in all were collected and put together in one cage for further experiments.

Aug. 21 (8 days after feeding on the infected guinea pigs). There were 83 mosquitoes surviving, and these were allowed to bite a normal guinea pig. All the mosquitoes sucked the blood eagerly. The protocol of this animal follows.

Guinea Pig 570.—Aug. 21. Bitten by 83 stegomyias. Temperature Aug. 22, 38.2°C.; Aug. 23, 38°; Aug. 24, 38.1°; Aug. 25, 39.8°; Aug. 26, 38°; Aug. 27, 37.2°; Aug. 28, 36.8°; Aug. 29, 37.6°; Aug. 30. 37°; Aug. 31, 36.2°; Sept. 2, 37.2°; Sept. 3, 36.5°. Death occurred during the night 13 days after the animal had been bitten.

Autopsy.—Jaundice general but mild; hemorrhages in the lungs, stomach, and intestines; liver yellowish brown; kidney intensely congested, containing some minute ecchymoses, yellowish bladder filled with yellowish brown urine with albumin and casts. Ecchymosis in the serosa and mucous membranes in general. Very few of the leptospiras in the kidney, none in the liver or blood.

Experiment 11.—To supplement the foregoing experiments, on Aug. 23, 1918, that is 2 days later, the same mosquitoes, twenty-five in all, were crushed in a mortar and emulsified in Ringer's solution. The emulsion was examined for the leptospira under the dark-field microscope and also smeared over the scarified surface of the skin of a normal guinea pig. Occasional specimens of leptospira were found in the emulsion after long search.

The guinea pig, No. 544 A, came down with the typical symptoms on Aug. 31; that is, 8 days after the inoculation. In the liver and kidney the leptospira was demonstrated in small numbers under the dark-field microscope.

This experiment conclusively proves that the leptospira was present in the body of the stegomyia mosquitoes which had been fed on the infected guinea pig and were capable of transmitting infection by their bite to another animal. The course of the infection produced by smearing the mosquito emulsion over the skin was more rapid than that caused by the bite.

Experiment 12 (*Negative*).—Some of the mosquitoes used in Experiment 10 which had caused a positive transmission of *Leptospira icteroides* after 8 days from the time of feeding on infected guinea pigs were kept another week. As already stated, twenty-five of these 83 mosquitoes were crushed on Aug. 23 for a supplementary confirmation (Experiment 11) of Experiment 10. Subsequently most of the remaining females laid eggs, and some perished. On Aug. 28, 7 days after Experiment 10, only thirteen were left. On that date a normal guinea pig (No. 633) was placed in the cage, and all the mosquitoes became engorged. The guinea pig was kept under daily observation for 13 days, but there was no suspicion of infection, and it was discarded on Sept. 10.

Experiment 13.—Aug. 11, 1918, 11 a.m. An infected guinea pig (Case 1 strain) showing the typical symptoms was placed in a mosquito cage. Twenty-four engorged females were collected and put into another cage. Aug. 14. Sixteen additional engorged females were also put into the cage, making the total 40. Aug. 21 (10 days later). A normal guinea pig was placed in the mosquito cage. The mosquitoes fed eagerly, all becoming engorged within 10 minutes. This animal (Guinea Pig 572) remained apparently well for 11 days. It was found dead on Sept. 2. At no time did the morning temperature exceed 38.4°C., which was about normal for this animal.

Autopsy.—A trace of jaundice throughout the body; liver highly degenerated and pale yellow; a few ecchymoses in the lungs; kidneys much congested. There was too scanty a quantity of urine in the bladder to examine for albumin. Diagnosis: probably a mild infection with *Leptospira icteroides.*

SUMMARY.

The foregoing experiments show that symptoms and lesions closely resembling those of yellow fever in man may be induced in guinea pigs by the bite of female stegomyias that have previously sucked the blood of a yellow fever patient or of an animal experimentally infected with *Leptospira icteroides*. With mosquitoes infected directly from a yellow fever patient the infectivity seems to become manifest after a longer period of incubation than with those infected with the animal blood. In the former, at least 12 days are said to be necessary before they become infectious, and this hypothesis seems to be borne out by the present experiment. On the other hand, the mosquitoes which were engorged with the infected blood of the guinea pig were found to be capable of transmitting the disease within 8 days after the feeding. This discrepancy may be explained by the fact that the number of leptospira existing in experimentally infected guinea pigs is far greater than that in human blood.

The frequency with which positive transmission by the stegomyia was obtained in both instances was very small indeed, in view of the number of mosquitoes employed. It appears that even under natural circumstances the percentage of mosquitoes that eventually become infected with the yellow fever microbe by sucking the blood may be very small. It has already been shown by previous investigators[2,5] that to transmit yellow fever from a patient to a non-immune person requires from 0.1 to 2 cc. of blood at the height of disease. According to my estimate a female stegomyia may take up 0.01 cc. or even less. Apparently a mosquito occasionally becomes infectious by taking up the one or two organisms which happen to be circulating in the peripheral blood of man, and it is these occasionally infected few which carry the disease. It is not difficult to realize the extent of ever increasing danger from a constant supply of the microbic virus which an endemic center or an epidemic of yellow fever can provide. One infected mosquito may mean many patients, and the life of such a mosquito is usually longer than that of the persons whom it fatally infects.

Finally, it is of interest to note that the development and maintenance of *Leptospira icteroides* are indispensably associated with the blood constituent, the serum, and this is amply supplied by the blood-

sucking insect. The organism is one of the most fragile of all the pathogenic parasites and cannot survive the concurrence of other less fastidious organisms such as bacteria. The comparatively aseptic body cavity of the stegomyia[9] furnishes a secure shelter for the parasite, which undoubtedly penetrates the zone of safety as soon as it is taken into the stomach of the insect. Unlike many other parasites this organism is capable of penetrating the intact skin or a bacteria-proof filter, and hence it is probably an easy matter for it to pierce the tissue of the visceral organs of the mosquito. Whether or not *Leptospira icteroides* can survive and multiply only in the body of *Stegomyia calopus* and not in other varieties or genera is yet to be determined.

Another interesting fact with regard to the extrinsic life of this organism is that it can multiply steadily at a temperature from 18 –37°C. The optimum temperature, at which it remains viable for many months, is 26°. The climate in most of the tropical countries offers optimum conditions both for *Leptospira icteroides* and for the mosquito which carries and nourishes it.

[9] This refers to the presence of bacteria and not certain higher plant parasites (yeast, moulds, etc.) or protozoa which have been occasionally found in stegomyia mosquitoes. These non-bacterial organisms may exert no adverse influence upon *Leptospira icteroides*.

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CHEMOTHERAPY OF TRYPANOSOME AND SPIROCHETE INFECTIONS.

BIOLOGICAL SERIES. I.

THE TOXIC ACTION OF N-PHENYLGLYCINEAMIDE-*p*-ARSONIC ACID.

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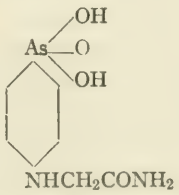
The chemotherapeutic investigations which have been in progress in these laboratories for several years have yielded a number of substances of striking activity in the treatment of experimental infections such as those produced in laboratory animals by various species of trypanosomes, the spirochetes of relapsing fever, and *Treponema pallidum*. Among the first of these substances was the amide of *N*-phenylglycine-*p*-arsonic acid, a description of which is given by Dr. Jacobs and Dr. Heidelberger, in the chemical series of these papers.[1] It may be said that while *N*-phenylglycine-*p*-arsonic acid, described in German Patent, No. 204,664, is a substance of practically no importance in the treatment of these infections, arsenophenylglycine, produced from it by reduction, was among the earliest of the highly active trypanocidal agents. The amide of this acid which has the structural formula



was first made and studied in the fall of 1915 with results which at once opened the way to the development of a number of important substances which will be dealt with in subsequent papers.

[1]Jacobs, W. A., and Heidelberger, M., *J. Exp. Med.*, 1919, xxx, 411.

Methods of Employing N-Phenylglycineamide-p-Arsonic Acid.

The amide of *N*-phenylglycine-*p*-arsonic acid, or A 63 as it was designated on our lists, presents no difficulty in the way of its use for purposes of animal experimentation. In the form of the monosodium salt, the drug is readily soluble in water in concentrations as high as 50 per cent and when injected directly into the tissues or into the body cavities of animals, it is readily absorbed and produces but slight irritation or local injury.

Preparation of Solutions.—Solutions of A 63 may be prepared by dissolving the monosodium salt in sterile distilled water, or in case the acid is used, the requisite amount of sodium hydroxide (0.37 cc. of N sodium hydroxide per 0.100 gm. of drug) to form the monosodium salt should be added slowly with stirring. The product then dissolves without difficulty.

Administration.—This drug may be administered to animals by almost any route which is convenient and has been used subcutaneously, intramuscularly, intraperitoneally, intravenously, and *per os* with no especial disadvantage following its use by any except the last named route.

Measurement of Doses.—The measurement of doses was accomplished in one of three ways. With the smaller animals, a stock solution of the drug was prepared from which individual doses for mice were measured by the use of a Fornier tuberculin syringe graduated in hundredths of a cubic centimeter; in the case of rats and guinea pigs, doses were measured with standardized pipettes. Finally, doses for larger animals such as rabbits and monkeys were always weighed and prepared separately. The values given in all cases refer to amounts of the monosodium salt which was used almost exclusively. These figures may be transposed into equivalents of the acid by the use of the factor 0.9 if desired.

Minimum Lethal Dose.

Although A 63 contains 24.57 per cent of arsenic, its toxicity for laboratory animals is comparatively low. The lethal dose of the drug varies between the extremes of 0.75 and 2.75 gm. per kilo of body weight for the different animal species in which it has been tested and for different routes of administration.

Mice.—The tolerance of mice is particularly good, as is indicated by the figures given in Table I which represents the combined results of several experiments carried out with mice. From the data at our disposal, we would place the minimum lethal dose for mice at 2.5 to 2.75 gm. per kilo of mouse when given subcutaneously, 2 to 2.25 gm. given intraperitoneally, and 2 gm. when given intravenously. These figures show a reasonably close agreement in the toxicity of the drug when administered by these routes, and the results obtained from successive experiments were comparatively uniform. Some mice survive even higher doses than those shown in the table, and conversely an occasional mouse may succumb to lower doses than those given as the lethal or minimum lethal dose; but we have had no

TABLE I.

Lethal Effects Obtained from the Administration of a 5 Per Cent Solution of N-Phenylglycineamide-p-Arsonic Acid to Mice in Doses Equivalent to 1.75 to 2.5 Gm. per Kilo of Mouse.

Dose per kilo.	Subcutaneous injection.		Intraperitoneal injection.		Intravenous injection.	
	No. of mice used.	No. died.	No. of mice used.	No. died.	No. of mice used.	No. died.
gm.						
2.5	2	0	5	1	2	2
2.25	4	0	21	5	4	1
2.0	4	0	25	2	4	1
1.75	4	0	8	0	4	0

deaths resulting from doses of the drug below 2 gm. per kilo of mouse.

Rats.—The resistance of white rats to the toxic action is lower and more irregular than that of any other animal with which we have worked. When the drug is administered intraperitoneally, doses as small as 0.75 gm. per kilo are sufficient to cause death in a fair percentage of animals and yet some rats will survive as much as 1.75 gm. per kilo. The results obtained from subcutaneous administration are distinctly better; the minimum lethal dose rises to 1 gm. per kilo and the action of the drug is definitely more constant, as may be seen by an examination of the results from the two experiments incorporated in Table II.

TABLE II.

Lethal Effects Obtained from the Subcutaneous Administration of a 10 Per Cent Solution of N-Phenylglycineamide-p-Arsonic Acid to Rats and from the Intraperitoneal Administration of a 3 Per Cent Solution.

Dose per kilo.	Subcutaneous injection.		Intraperitoneal injection.	
	No. of rats used.	No. died.	No. of rats used.	No. died.
gm.				
1.50	4	4	5	4
1.25	4	1	5	3
1.00	9	6	9	7
0.90	5	0	5	2
0.75	5	0	5	3
0.60			5	0
0.50			5	0

Guinea Pigs.—Contrary to the opinion which is generally held as to the tolerance of guinea pigs for arsenicals, these animals withstand relatively large doses of A 63 and the resistance of individual animals appears to be fairly uniform. The lethal dose found for the drug was 1.5 gm. per kilo of body weight whether given subcutaneously or intraperitoneally (Table III).

TABLE III.

Lethal Effects Obtained from the Administration of a 20 Per Cent Solution of N-Phenylglycineamide-p-Arsonic Acid to Guinea Pigs.

Dose per kilo.	Subcutaneous injection.		Intraperitoneal injection.	
	No. of guinea pigs used.	No. died.	No. of guinea pigs used.	No. died.
gm.				
1.75			4	1
1.50	4	3	9	3
1.40	4	0	4	0
1.25	4	0	4	0

Rabbits.—A great deal of time has been devoted to the study of the toxic action of A 63 in rabbits. The major part of this work was carried out by intravenous administration of the drug but the effects

of subcutaneous, intramuscular, and *per os* administrations have all been investigated to some extent. The solutions used in the experiments varied between concentrations of 5 and 50 per cent, partly for the purpose of studying the effects of the use of different volume doses and partly for the purpose of determining the influence of such factors as rate of administration and concentration of solutions upon the action of the drug.

It was found that as much as 20 cc. per kilo of a 5 to 10 per cent solution could be injected intravenously into rabbits about as rapidly as one wished, and that in general, solutions of low concentration (5 to 10 per cent) were better borne than those of a higher concentration. The effects of concentration were less evident, however, when the drug was administered by other routes, and more concentrated solutions were employed as a means of reducing the volume of fluid which had to be used.

Under the conditions described, the minimum lethal dose for rabbits was found to be 0.75 to 0.9 gm. per kilo of body weight when given intravenously and 1.1 gm. given either subcutaneously or intramuscularly, with lethal effects as indicated by the experiments recorded in Table IV.

TABLE IV.

Lethal Effects Obtained from the Intravenous Administration of a 5 to 10 Per Cent Solution of N-Phenylglycineamide-p-Arsonic Acid to Rabbits and from the Subcutaneous or Intramuscular Administration of 50 Per Cent Solutions.

Dose per kilo.	Subcutaneous injection.		Intramuscular injection.		Intravenous injection.	
	No. of rabbits used.	No. died.	No. of rabbits used.	No. died.	No. of rabbits used.	No. died.
gm.						
1.25	8	6	4	3		
1.1	4	4	4	2		
1.0	3	0	3	0	6	3
0.9					12	4
0.75					10	1

Our experience in giving this drug to rabbits by mouth may be recited very briefly. The drug was given in solution by means of

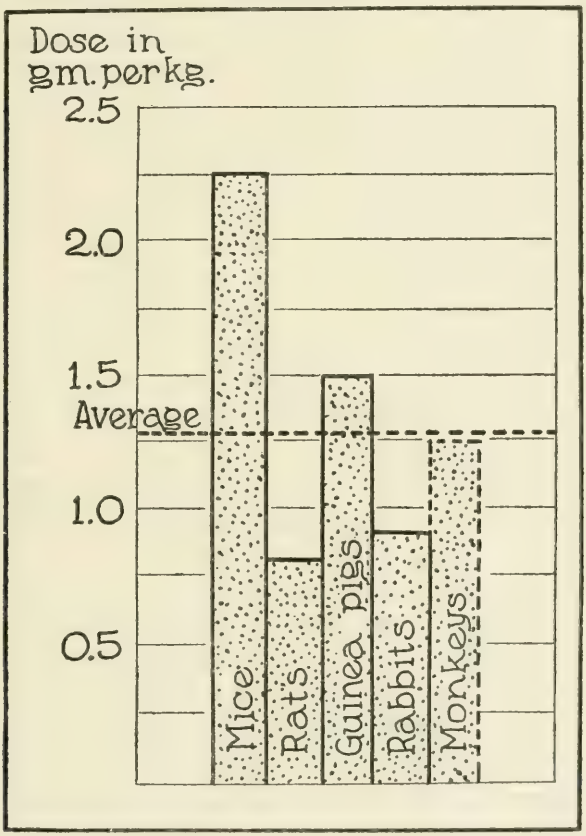
a stomach tube following the administration of a small dose of bicarbonate of soda. In all, there were only seven rabbits which received the drug in this way. Two of them were given doses of 1.25 gm. per kilo of body weight, four were given 1 gm., and one was given 0.75 gm. The last of these animals showed no ill effects from the drug and only two of the others actually succumbed to its action, both of which received a dose of 1 gm. per kilo. One of these rabbits lived 4 days and the other 24 days after the administration of the drug. It appeared certain, however, that none of the other animals would recover completely and they were killed at different times for purposes of pathological examination. As the matter stands, therefore, we are hardly justified in attempting to fix the toxic limits of A 63 when given *per os*.

Monkeys.—Lastly, we have used two monkeys (*Macacus rhesus*) in studying the toxic action of A 63. The first monkey, a female weighing 2,325 gm., was given an initial dose of 0.75 gm. per kilo of body weight. The animal was observed for 5 days during which time no ill effect from the drug could be detected, and a second dose of 1 gm. per kilo was given with a like result. 4 days later, the animal received its third dose which was 1.25 gm. per kilo. Following this dose, the only evidence of intoxication noted was possibly a slight loss of weight (75 gm.). At the end of 3 weeks, the weight of the animal was 2,450 gm., and a fourth dose of the drug was given—this time 1.5 gm. per kilo, or double the initial dose. The monkey showed slight signs of intoxication lasting for a few days, followed by rapid recovery. The animal was kept under observation for 3 months and was then killed for pathological purposes.

The second monkey used was likewise a female *rhesus* weighing 2,650 gm. This monkey was given an initial dose of 1.25 gm. per kilo intravenously. There was a loss of weight in this animal amounting to 175 gm., but no other evidence of intoxication developed, and after waiting 1 week, a second dose of 1.5 gm. per kilo was given; death followed within 24 hours.

These two experiments are cited merely to show that monkeys possess a degree of tolerance for A 63 comparable with that of other animals and that they react to the drug in much the same way.

Represented graphically, the values obtained for the minimum lethal dose of this drug in the five species of animals studied would form a curve such as that given in Text-fig. 1. While these values are not strictly comparable on account of differences in the mode of



TEXT-FIG. 1. Comparative magnitudes of the minimum lethal dose of *N*-phenylglycineamide-*p*-arsonic acid for different animal species. The dose given for monkeys is an approximate estimate.

administration used, they will serve to indicate the relative magnitude of the toxic dose for different animal species. The mean toxic dose for this group of animals is found to be approximately 1.28 gm. per kilo; the maximum variation from this mean is as 1: 1.75 and the extremes compare as 1: 2.8.

Symptoms and Course of the Intoxication.

The symptom-complex of the intoxication produced by *N*-phenylglycineamide-*p*-arsonic acid in laboratory animals is characterized by two groups of phenomena, one nervous and the other nutritional in character. The most prominent of these symptoms appear at an early period of the intoxication, as pronounced tremors with incoordination of movements, or in extreme cases, as clonic spasm, usually associated with some weakness or complete prostration. With remission of these early symptoms in mice, a tic develops which is characterized by peculiar jerky movements of the head and occasionally by the continuous circling movements of dancing mice. Nutritional disturbances are indicated chiefly by a loss of appetite, more or less weakness, loss of weight, and occasionally by diarrhea. Altogether the picture is one previously recognized and described as characteristic of the toxic action of a number of pentavalent arsenicals, particularly of arsacetin, dichlorophenolarsonic acid, and aminohydroxyphenylarsonic acid.

These symptoms are by no means constant either as to the frequency or the intensity of their occurrence in different animal species or in individual animals of the same species.

Mice.—In mice symptoms of intoxication occur only following relatively large doses of the drug (1.75 gm. per kilo and above) and even then are not of constant occurrence. In the most marked cases, mice show violent muscular tremors on being disturbed and marked locomotor incoordination. After 2 or 3 days, these symptoms disappear entirely or gradually give place to the tic described, which in turn rarely persists for more than a week. Disturbances of this general character appear to be more pronounced following subcutaneous and intravenous administrations of the drug than after intraperitoneal administration.

Mice also show some nutritional disturbances for 2 or 3 days following toxic doses of the drug but make a rapid recovery with an increase in weight above their normal level and remain subsequently in excellent condition.

Rats.—Rats are, on the whole, the most sensitive animals to this drug and individual idiosyncrasy is quite pronounced among them.

Nevertheless, toxic symptoms in these animals are fairly well confined to doses within what we regard as the lethal range. While some rats show no toxic symptoms at all, in others, tremors, incoordination, convulsions, weakness, and emaciation reach a most extreme grade and progress to a lethal termination, recovery under such conditions being very rare.

Guinea Pigs.—Guinea pigs on the other hand are quite resistant to A 63. They show very slight evidence of intoxication either of a nervous or of a nutritional character from doses below the lethal level. The dose that is survived is usually well borne and even lethal doses produce comparatively slight tremors, unsteadiness, weakness, or loss of weight.

Rabbits.—The reaction of rabbits to A 63 is rather irregular. By whatever route the drug is administered, doses above 0.5 to 0.6 gm. per kilo of body weight cause some loss of appetite with an initial loss of weight which is in proportion to the size of the dose used. With doses of 0.75 to 0.9 gm. per kilo of body weight, the loss of weight in surviving animals may reach as much as 200 to 300 gm. and is associated with some degree of weakness. This loss of weight, however, is quickly regained, leaving no symptomatic evidence of intoxication. Nervous phenomena of the exact type described in mice and rats do not occur in rabbits. Instead, rabbits manifest some hypersensitiveness in the milder cases of intoxication, while in extreme cases incoordination develops with a tendency to clonic spasms, and there is eventual loss of muscular control or even paralysis. As in the case of the rat, pronounced nervous symptoms in rabbits nearly always portend a lethal outcome so that these phenomena cannot be said to occur as symptoms of a sublethal intoxication.

When toxic doses are given to rabbits *per os*, the drug produces symptoms which are not observed when it is given by other routes, the characteristic feature of which is a marked abdominal distention with flatulence as a result of atony of the colon. Associated with this condition, there is a loss of appetite, diarrhea, progressive loss of weight, and weakness. The condition is extremely persistent and recovery, if it occurs at all, is problematical.

Monkeys.—In our limited experience with monkeys, we have observed no symptoms of intoxication other than slight loss of weight and some weakness as previously recorded.

Having described the symptoms of intoxication produced by A 63, we shall now take up the question of the time element in these toxic reactions. In mice symptoms of intoxication are usually apparent within 24 hours after the administration of the drug but may be delayed until the 2nd day. Death also occurs relatively early in these animals and is rare after 48 to 72 hours. Symptomatic recovery is relatively prompt.

Rats, on the other hand, usually show no symptoms of intoxication until the 2nd or 3rd day except with very large doses of the drug. Out of twenty-eight rats that died as a result of intoxication with doses below 1.5 gm. per kilo of body weight, the earliest death occurred 3 days after the administration of the drug, the latest deaths 10, 11, and 14 days, while the average period was 6 days. Recovery in rats is also a slow and uncertain process.

Symptoms of intoxication in guinea pigs usually make their appearance on the 2nd day following the administration of the drug. Among the deaths recorded in our series from doses below 2 gm. per kilo, the earliest occurred 2 days and the latest 7 days after the administration of the drug with an average period of 4 days survival. Recovery in guinea pigs is usually prompt and complete.

Of the rabbits which died after intravenous injections of a single dose of A 63, one with the smallest dose (0.75 gm.) died in almost exactly 24 hours, while the longest survival was 6 days and the average 2.6 days. The minor symptoms of intoxication in rabbits are not apparent as a rule until the 2nd day, while the graver symptoms occur more promptly. As previously indicated, symptomatic recovery in the rabbit is usually prompt and complete.

Pathology of the Intoxication.

The pathological changes produced in the animal organism by toxic doses of A 63 constitute an important phase of its toxicologic action. Viewed from the standpoint of the bearing of these changes upon the possible usefulness of the drug as a therapeutic agent, this phase of the subject resolves itself largely into a consideration of organic injury and recovery therefrom.

Local Effects.

As we have already indicated, the injury produced by the drug at the site of injection is almost negligible. Full toxic doses may be injected into the veins of animals even in saturated solution, with practically no local reaction. When a 50 per cent solution of the drug is injected into the subcutaneous tissues of animals, a slight edema develops at the site of the injection but clears up almost immediately, leaving such slight evidences of tissue reaction as are barely recognized from external examination. If given intramuscularly in amounts small enough to obviate mechanical laceration of the tissues, the reaction which follows is likewise very mild and is of essentially the same character as that which follows subcutaneous administration.

Systemic Effects.

The effects produced by the drug upon the organism as a whole are of much more importance than those of a purely local character. These effects are divisible into major and minor phases of pathological action, the details of which differ somewhat in different animal species as well as with the amount of the drug used and the route of administration employed. In general, however, the changes seen during the early stages of the intoxication consist in moderate vascular dilatation and congestion with a few scattered petechial hemorrhages, occasional effusions into the serous cavities, and widespread cellular degenerations or even necrosis in some organs. The organs which share most prominently in these changes are the kidneys and adrenals, and the cardiovascular system, with an uncertain involvement of the central nervous system, the gastrointestinal tract, the blood, and blood-formative organs; the changes produced in other organs appear to be of minor importance. Animals which survive the intoxication rarely show lesions in organs other than the kidneys and myocardium.

Kidneys.—The central feature of the pathological action in all animals is the injury to the kidneys. In acute poisoning, the kidneys are somewhat enlarged and tend to be pale except for the presence of more or less congestion or even hemorrhage in the boundary zone

which may extend outward along the medullary rays to the capsular surface. The glomerular tufts are usually swollen, the capillaries congested, and the covering epithelium is degenerated. Degeneration or even necrosis of the tubular epithelium is rather widespread but occurs chiefly in the ascending loops of Henle and the convoluted tubules, particularly those located along the medullary rays and the outer portion of the cortex. The interstitial elements of the kidney are, as a rule, much less affected except when overwhelming doses of the drug are given.

As serious as these injuries appear to be in the acute stages, recovery is remarkably satisfactory in most animals. Reaction of the tissue elements sets in promptly and restoration of the injured parts is accomplished within a short period of time and with comparatively slight scarring or distortion of the architecture except where the initial destruction was unusually extensive. In these cases, the marks of injury are found in a growth of connective tissue and in round celled infiltrations extending along the medullary rays and through the outer portion of the cortex, while a few glomeruli show shrunken tufts and thickened capsules, all of which gives a granular surface to the kidneys.

Adrenals.—Evidences of injury to the adrenals consist in swelling, with some congestion of the cortical vessels and the occasional presence of focal hemorrhages in the cortex. In guinea pigs, the later changes are manifested by a decrease in the cortical pigmentation with well marked degenerative changes in the cortex and medulla or even necroses in the midcortical zone. Here again recovery appears to take place fairly promptly.

Cardiovascular System.—The most pronounced effects of A 63 upon the cardiovascular system appear during the early stages of the intoxication. There is a moderate vascular dilatation and congestion throughout the body and a few petechial hemorrhages may occur in any of the organs. The sites of chief importance are the kidneys, the adrenals, and heart in which occasional foci of hemorrhage are found beneath the epicardium and the endocardium as well as in the myocardium itself. In several instances, hemorrhages from the meningeal vessels have been observed, either in the region of the torcular or in the retroorbital tissues.

Animals which survive the acute poisoning show little evidence of either congestion or hemorrhage, but in their stead one finds an accumulation of more or less fluid in the serous cavities, perirenal edema, and in rare instances an increased tension of the cerebrospinal fluid. These conditions we regard as at least indicative of vascular injury. In addition to such changes as these, the myocardium shows a moderate fatty degeneration with occasional areas of fibrosis. On the whole, the pathological effects of A 63 upon the cardiovascular system are not pronounced and the conspicuous congestion, hemorrhage, and degeneration so often observed with arsenicals are largely absent from the changes which characterize the action of this drug.

Central Nervous System.—From the symptoms of the toxic action of A 63, one might expect the central nervous system to share the position of chief pathological importance with the kidneys and cardiovascular system. Such changes as occur, however, are rather obscure and difficult of determination in laboratory animals. We have already mentioned the occurrence of congestion of the meningeal vessels and occasional hemorrhages together with an increased tension of the cerebrospinal fluid. Histologically, the chief evidences of injury are associated with the choroid plexus and with the small and medium vessels of the meninges and brain. The plexus itself shows definite degeneration in some animals and the tissues immediately surrounding the vessels are somewhat edematous. Apart from these changes, however, we have been able to demonstrate only minor degrees of cellular degeneration of a rather indefinite character and of uncertain extent.

Blood and Blood-Formative Organs.—While the action of A 63 upon the blood and blood-formative organs forms an important feature of the effect of the drug, this action is perhaps more physiological than pathological and hence there is little that can be said in the present connection.

Poisoning with the drug produces an initial destruction of red blood cells and an accumulation of blood pigments in the spleen, liver, and bone marrow. The formative centers, especially those of the spleen, show degeneration or even necrosis after the administration of very large doses of the drug, but following these changes, there is an extremely active hyperplasia in which all elements participate.

This is most strikingly shown by a myeloid metaplasia in the spleen of rats and mice which is characterized by the presence of large numbers of megacaryocytes. These changes alone, however, might mean very little, since such reactions are easily excited in mice and rats.

The effect of A 63 upon other organs of the body is relatively inconsiderable. In the liver, parenchymatous and fatty degeneration are usually but not constantly present, and in exceptional instances isolated cells or groups of cells here and there show necrosis. As a rule, these changes clear up very quickly leaving nothing to indicate the existence of any previous injury.

Lesions of the gastrointestinal tract are likewise inconstant and of uncertain significance. During the early stages of the intoxication, a catarrhal condition of the entire tract is not infrequently observed but is rarely of serious degree. An exception to this rule is found in animals which have been given the drug by mouth. These animals always show a pronounced and persistent colitis with a lesser degree of involvement of the stomach and small intestine. This, however, is clearly referable to the mode of administration of the drug.

Apart from a knowledge of the character of the lesions which may be induced by this drug, the features of chief interest from the pathological point of view are the promptness with which repair is accomplished and the fact that, as the dose of the drug used falls below the level of the lethal dose, the extent of the resulting injury rapidly diminishes until, with doses only slightly below the level of the lethal dose, organic injury is barely demonstrable. These features of the pathological action of A 63 are both unusual and of considerable practical importance in their bearing upon the use of the drug for therapeutic purposes.

Tolerance of Repeated Doses.

The facts presented thus far have dealt entirely with effects produced by the administration of single doses of the drug, which brings us to a consideration of the reaction of the animal organism to the use of repeated large doses. This phase of drug action is bound up to a large extent with two conditions, first, the time during which the drug remains in the animal body in a form capable of exercising a

toxic effect, and second, the character and duration of the effect produced, whether functional or organic.

In attempting to arrive at some idea of the time during which A 63 remained biologically active after being administered to animals, we made use of two types of experiments—one based upon protection against infection and the other upon superposition of fractional parts of toxic doses. In general, it was found by experiments of the first class, that the protection afforded such animals as mice against infection with *Trypanosoma brucei* was practically nil 24 hours after the administration of as much as twice the dose of the drug which was capable of curing a 24 hour infection of the same organism (0.5 gm. per kilo). With rats and with rabbits on the other hand, some degree of protection still existed at the end of 48 hours. In the case of the rabbit, four animals which were inoculated with *Trypanosoma brucei*, 48 hours after having received an intravenous injection of 0.5 gm. of A 63 per kilo, gave the following results: One rabbit showed no protection, two were permanently protected, and the fourth showed an incubation period of 21 days as contrasted with an incubation period of 9 days in controls which received the same dose of organisms at the same time.

From these experiments it appeared that retention of the drug in a biologically active form was a variable condition both specifically and individually, but that in animals such as the rat and the rabbit, there was the possibility of the drug's remaining in the body for 48 hours or even longer in a condition in which it might still be capable of exerting toxic effects. To test this further, we carried out a few experiments on the effects of the administration of fractional parts of a toxic dose at various intervals of time. Without going into the details of these experiments, we may say that it was found that when the toxic dose of A 63 for rabbits (0.75 gm. per kilo) was divided into three equal parts administered at intervals of 24 hours, the toxic effect approached that produced by the administration of the entire amount of drug at one time. This, of course, might be interpreted either as evidence of an accumulation of drug or as evidence of superposition of effects notwithstanding the fact that no toxic effect could be recognized from the administration of a single such fractional part of the toxic dose.

Combining these facts with what we had already learned in regard to the general reaction of animals to single doses of A 63, we undertook some experiments intended to determine the tolerance of mice and of rabbits to prolonged repetition of large doses of the drug and something of the interval at which such repetitions might be carried out successfully. The results obtained with one series of mice are summarized in Table V.

These mice were given an initial dose of 2 gm. of A 63 per kilo of body weight injected into the peritoneal cavity. The injections were repeated at weekly intervals and the dose was progressively increased up to 3 gm. per kilo. The sixth and last dose of 2.5 gm. per kilo was

TABLE V.

Tolerance of Mice to Intraperitoneal Administrations of Increasing Doses of N-Phenylglycineamide-p-Arsonic Acid Given at Weekly Intervals.

No. of injection.	Dose per kilo.	No. of mice used.	No. intoxicated.	No. died.
	gm.			
1	2.00	15	5	2
2	2.25	13	0	0
3	2.50	13	2	2*
4	2.75	11	0	0
5	3.00	11	0	0
6†	2.50 (i.v.)	9	3	3

* These two mice were toxic after the first dose.

† The last injection was given intravenously.

then given intravenously. Five of the fifteen mice showed symptoms of intoxication following the administration of the first dose and two of these died. Two others died after receiving the third dose (2.5 gm.) but the remaining eleven mice survived the administration of 3 gm. per kilo with no evidence of intoxication, indicating a definite increase in their tolerance to intraperitoneal administrations of the drug. When the route of administration was changed, these animals still showed a resistance to the drug slightly greater than that of normal mice.

Experiments of a similar character were carried out with rabbits. In this case, three routes of administration were used—subcutaneous, intramuscular, and intravenous. The initial doses were placed at

what was regarded as comparable levels for the various routes of administration; *i.e.*, 0.75 gm. per kilo intravenously and 1 gm. per kilo given either subcutaneously or intramuscularly. Animals of the subcutaneous and intramuscular series received their last dose intravenously and those of the intravenous series were divided into two

TABLE VI.

Tolerance of Rabbits to Increasing Doses of N-Phenylglycineamide-p-Arsonic Acid Given at Weekly Intervals, (A) Subcutaneously, (B) Intramuscularly, and (C) Intravenously.

No. of injection.	Dose per kilo.	No. of rabbits used.	No. intoxicated.	No. died.
	gm.			
A 1	1.00	3	0	0
2	1.00	3	0	0
3	1.10	3	0	0
4	1.25	3	0	0
5	1.35	3	0	0
6*	1.10	3	0	0
B 1	1.00	3	0	0
2	1.00	3	0	0
3	1.10	3	0	0
4	1.25	3	0	0
5	1.35	3	0	0
6*	1.10	3	0	0
C 1	0.75	10	2	1
2	0.75	9	1	0
3	0.90	9	0	0
4	1.00	9	1	1
5	1.10	8	0	0
6*	1.35	8	1	1

* The last injections of Series A and B were given intravenously; in Series C, four rabbits were injected subcutaneously and four intramuscularly.

groups one of which was given the last dose subcutaneously and the other intramuscularly. The results of these experiments are given in Table VI.

Following the first dose, one rabbit of the intravenous series was extremely toxic and died within 24 hours. A second rabbit was slightly toxic but all the others remained in good condition. No

other toxic manifestations developed until the fourth dose of the series was given, when one rabbit of the nine which received the dose of 1 gm. per kilo intravenously became toxic and died after 17 days. The fourteen remaining rabbits were carried through to the conclusion of the experiment with no symptoms suggestive of a harmful effect other than slight fluctuations in weight.

The dose in the intravenous series was raised progressively from 0.75 gm. per kilo of body weight to 1.1 gm., which in our experience is almost uniformly fatal when given to normal rabbits, with the loss of only two out of the ten rabbits; the eight rabbits which received the dose of 1.1 gm. survived with no evidence whatsoever of intoxication. These eight rabbits were then given a dose of 1.35 gm. of A 63 per kilo of body weight either subcutaneously or intramuscularly with one death following an intramuscular injection. The other rabbits remained entirely normal and continued to gain weight. With the subcutaneous and intramuscular series, the dose was raised from 1 gm. per kilo of body weight to 1.35 gm. with no toxic developments, and all these animals were then given 1.1 gm. of the drug by the intravenous route. In this case, the weights declined slightly but there was no other sign of intoxication. It is to be noted that all three groups of rabbits were carried to a point where they not only survived doses which are ordinarily fatal but that when a normally lethal dose was given by a route other than that to which they had been accustomed, only one of the fourteen rabbits succumbed, while the others showed little or no ill effects.

At the conclusion of this experiment, the entire group of rabbits was killed for pathological examination. On opening these animals, there was a strong odor of garlic—a condition never noted in an animal which had died from a single dose of the drug. Pathologically, the only changes found were small subcutaneous abscesses or abscesses in the lumbar muscles of animals which had received repeated injections in the same regions, a slight fatty degeneration of the myocardium with a few patches of fibrosis, and chronic nephritis. The injuries to the heart muscle and to the kidneys were definitely more pronounced in the rabbits of the intravenous group than in the others. With reference to the kidneys of this group, we would classify them as two with slight lesions, two with moderate lesions, and four with pronounced lesions.

The total amount of drug received by each of these rabbits during a period of 36 days was 5.85 gm. per kilo in one group and 6.8 gm. per kilo in the other, or 1.437 to 1.67 gm. of arsenic administered within a period of 5 weeks. Finally, it should be recalled that the toxic dose of A 63 for rabbits is lower than that for any other animal with which we have worked except the rat, and that the mice previously referred to received more than twice these amounts of the drug.

Our limited experience with monkeys, as previously stated, merely served to indicate that under properly chosen conditions, they too would respond to repeated doses in essentially the same way as rabbits or mice. While we do not know what the lethal dose of this drug is for monkeys, it is probably not less than 1 to 1.25 gm. per kilo of body weight, or approximately the same as that for guinea pigs and considerably more than that for the rabbit. In the one instance in which gradually increasing doses of A 63 were given to a monkey, we succeeded in giving as much as 1.5 gm. per kilo as the final dose with very slight intoxication resulting. When this animal was killed 3 months later for pathological examination, the organs appeared normal.

CONCLUSION.

The essential facts to be gathered from these studies of the toxicologic action of *N*-phenylglycineamide-*p*-arsonic acid may be summarized very briefly. The substance is one which lends itself well to almost any method of administration and can be given to animals in very large doses. The tolerance of different animal species varies rather widely but with one exception the reaction of laboratory animals to toxic doses of the drug is of favorable character. That is, toxic effects are confined to doses relatively close to the minimum lethal dose and the recovery of animals from sublethal intoxications is remarkably rapid and complete. This feature of the action of the drug makes possible the repeated administration of even very large doses at comparatively short intervals of time without incurring the dangers incident to cumulative action or to superposition of toxic effects. On the contrary, by taking advantage of this peculiarity of action, it is possible to develop such a degree of tolerance on the part of animals that the dose of the drug administered can be progressively

increased to a point well above that which is fatal to the normal animal, and this stands out as the feature of the toxicologic action of *N*-phenylglycineamide-*p*-arsonic acid which is of greatest significance in the use of the drug for therapeutic purposes.

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CHEMOTHERAPY OF TRYPANOSOME AND SPIROCHETE INFECTIONS.

Biological Series. II.

The Therapeutic Action of N-Phenylglycineamide-*p*-Arsonic Acid in Experimental Trypanosomiasis of Mice, Rats, and Guinea Pigs.

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The arsenical compound *N*-phenylglycineamide-*p*-arsonic acid, the toxic action of which has been described in the preceding paper, exercises a powerful therapeutic effect in experimental trypanosomiasis of laboratory animals. Trypanosomiasis as it occurs in mice and rats is chiefly characterized by the constant and progressively increasing number of trypanosomes in the peripheral blood stream, by the usual lack of any clinical manifestations, and by the relatively early death of the infected animal. An effective therapeutic compound, therefore, for the treatment of trypanosomiasis of mice and rats must be biologically available within a very short time after its administration and must have a sufficient speed and duration of action to halt and eventually overcome a rapidly increasing blood infection comparable, in part, with a bacteriemia, which if not checked, will cause the death of the animal in a few days or even hours. On the other hand, trypanosomiasis of many of the larger animals, especially of the rabbit, is preeminently a tissue infection and is characterized, in the acute stages, by conspicuous edematous and inflammatory swellings of the soft parts, particularly of the head and external genitalia, together with loss of appetite, weakness, and emaciation, while in the more chronic phases of the infection, the inflammatory lesions undergo induration and even necrosis with involvement of the deeper tissues including the periosteum and bone. The duration of the infection in

rabbits is a matter of weeks or months and the presence or absence of trypanosomes in the blood stream is distinctly of minor importance. It is obvious that a drug which is used for the treatment of this type of infection must possess in addition to trypanocidal action, the power of penetrating diseased tissue, and since this may require a considerable amount of time, the drug in question must remain biologically active in the animal host as long as may be necessary.

In order, therefore, to arrive at a full appreciation of the therapeutic action of a drug in experimental trypanosomiasis, one has, from the point of view of the animal factor, two general types of infection, the treatment of which will furnish information as to the speed of action on the one hand and as to the duration of action or potency of the drug on the other. This information, which should include such data as the determination of a therapeutic range in different animal species as well as curative doses, the therapeutic action against different species of trypanosomes, the comparative value of different routes of administration, and the results of a repeated dose system of therapy, together with the data upon the toxicological and pathological action of the drug in question in various animal species, furnishes a logical foundation for an accurate appraisal of the action of the drug under experimental laboratory conditions and, at the same time, furnishes a basis for estimating its probable value in the treatment of trypanosomal infections as they occur in nature.

The present paper includes the therapeutic results obtained with the amide of *N*-phenylglycine-*p*-arsonic acid in experimental trypanosomiasis of mice, rats, and guinea pigs. While trypanosomiasis of guinea pigs is, strictly speaking, a chronic infection of cyclic character, the results of its treatment with this drug are incorporated with those of acute infections of mice and rats.

EXPERIMENTAL.

Strains of *Tr. brucei*, *Tr. gambiense*, *Tr. equinum*, *Tr. equiperdum*, and *Tr. evansi*, carried constantly in stock mice and stock guinea pigs, were used for the infecting organisms, and all inoculations were made intraperitoneally. The details of animal inoculation for therapeutic purposes and the general conduct of such experiments have been de-

scribed[1] and need not be repeated here; but in this connection, mention may be made of the desirability of regulating and maintaining a uniform grade of infection, a factor of importance in interpreting therapeutic results. In the majority of experiments, we used a strain of *Tr. brucei* which is highly virulent and uniformly fatal for mice, rats, and guinea pigs, mice dying in from 60 to 70 hours, rats in from 4 to 5 days, and guinea pigs on an average of 4 weeks after the usual intraperitoneal inoculation. With organisms of less virulence, the size of the inoculating dose was proportionately adjusted to insure an infection comparable with that produced by our strain of *Tr. brucei*.[2] Both mice and rats were treated 18 to 24 hours and guinea pigs usually 1 week after inoculation, after parasites had been demonstrated in the peripheral blood. All the untreated controls died irrespective of the species of trypanosome used. Daily examinations of the blood were made in the mouse experiments for the 1st week or 10 days and at frequent intervals thereafter, while with rats and guinea pigs, the examinations usually began with 2 day intervals. The treated animals were kept under observation from 1 to 3 months.

Doses of the drug used are expressed in grams of the sodium salt per kilo of body weight. The solutions were always freshly prepared with sterile distilled water in various concentrations (0.5, 1, and 2 per cent) and the intraperitoneal route of administration was usually employed. The concentration of the drug solution in any given experiment remained a constant, the volume of the dose being the variable quantity.

Treatment of Experimental Trypanosomiasis of Mice.

For convenience of comparison, we have summarized, in the accompanying tables, the various protocols of individual experiments which were undertaken for the purpose of ascertaining the range of therapeutic action of the drug. We have included under the heading "intercurrent deaths" those animals which died from accidental

[1] Pearce, L., and Brown, W. H., *J. Exp. Med.*, 1918, xxviii, 109.

[2] In the case of *Tr. equinum*, the infection produced in several mouse and rabbit experiments was of an unusually severe nature, a condition which may be referable to an increase in the virulence of the strain at the time of inoculation or to the strength of the inoculating dose employed.

causes within the first 15 days after treatment in the cases of *Tr. brucei*, *Tr. gambiense*, and *Tr. equinum*, and within the first 30 days in the cases of *Tr. equiperdum* and *Tr. evansi*, since it was found that relapses practically always occurred within these periods of time with our strains of these species of trypanosomes, and none of the animals listed under "intercurrent deaths" is included in the number of cured animals irrespective of the size of the dose administered.

Trypanosoma brucei.—Table I shows the results of the treatment of *Tr. brucei* infection in 75 mice with graded doses of the amide of

TABLE I.

Tr. brucei Infection of Mice Treated with N-Phenylglycineamide-p-Arsonic Acid 24 Hours after Inoculation. Intraperitoneal Administration.

Dose per kilo.	No. of mice.	No. of intercurrent deaths.	No. of relapses.	No. of cures.
gm.				
1.0	2	0	0	2
0.9	2	0	0	2
0.75	2	0	0	2
0.6	3	0	0	3
0.5	1	0	0	1
0.4	2	1	0	1
0.35	1	0	0	1
0.3	11	1	0	10
0.275	5	0	0	5
0.25	19	1	2	16
0.225	13	0	3	10
0.2	13	0	8	5
0.15	1	0	1	0
Total.....	75			

N-phenylglycine-*p*-arsonic acid ranging from 0.15 to 1 gm. per kilo of body weight administered intraperitoneally. The majority of animals were treated with doses of from 0.2 to 0.3 gm., since doses above this level seemed uniformly curative, and in fact, no relapse was demonstrable with amounts of 0.275 gm. or more. Two mice, one treated with 0.4 gm. and the other with 0.3 gm., died from accidental causes within the first 15 days and consequently were not included in the number of cured animals, although in neither case could trypano-

somes be demonstrated. All the other mice which were treated with doses ranging from 0.275 to 1 gm. showed no relapse and all lived 30 days after treatment. Doses of 0.2, 0.225, and 0.25 gm. per kilo of body weight were found to exert a definite therapeutic action, although at these levels relapses occurred. With 0.25 gm., two out of nineteen mice relapsed and one died from intercurrent causes; with 0.225 gm., three relapses occurred out of thirteen mice; with 0.2 gm., there were eight relapses out of thirteen treated animals. With doses at this level, relapses usually appeared from 3 to 9 days after treatment. In some instances with doses of 0.2 and 0.225 gm., parasites were never completely cleared from the blood but they were definitely less numerous than in the blood of the untreated controls and in addition, these mice lived longer than the controls.

TABLE II.

Tr. brucei Infection of Mice Treated with N-Phenylglycineamide-p-Arsonic Acid 24 Hours after Inoculation. Intravenous Administration.

Dose per kilo.	No. of mice.	No. of intercurrent deaths.	No. of relapses.	No. of cures.
gm.				
0.6	1	0	0	1
0.5	1	0	0	1
0.4	1	0	0	1
0.3	1	0	0	1
0.25	2	0	0	2
0.2	1	0	0	1
0.15	1	0	1	0
0.1	1	0	1	0
0.05	1	0	1	0
0.025	1	0	1	0
Total.....	11			

The number of mice treated intravenously and subcutaneously, shown in Tables II and III, is relatively small, yet the therapeutic results by such routes are in striking accord with those obtained by intraperitoneal administration of the drug. Of eleven mice treated intravenously with doses ranging from 0.025 to 0.6 gm. per kilo, four animals which were given doses of 0.025, 0.05, 0.1, and 0.15 gm. respectively were not cured and the blood of those receiving the three lowest doses was never cleared of parasites, while the mouse

which was treated with 0.15 gm. relapsed on the 4th day. Doses of 0.2 gm. and above were uniformly curative as contrasted with the slightly higher level of 0.275 gm. by intraperitoneal administration.

Fourteen mice were treated subcutaneously, as indicated in Table III, with doses of 0.2, 0.25, and 0.3 gm. with no relapses, although there was one intercurrent death in the first fortnight which is not

TABLE III.

Tr. brucei Infection of Mice Treated with N-Phenylglycineamide-p-Arsonic Acid 24 Hours after Inoculation. Subcutaneous Administration.

Dose per kilo.	No. of mice.	No. of intercurrent deaths.	No. of relapses.	No. of cures.
gm.				
0.3	4	0	0	4
0.25	5	1	0	4
0.2	5	0	0	5
Total.....	14			

included in the number of cures. From these results, it would seem that both intravenous and subcutaneous routes of administration of the drug in *Tr. brucei* infections of mice were equally efficacious, since in both the dose of 0.2 gm. per kilo of body weight was uniformly curative, a dose which represents the lower limit of therapeutic action by intraperitoneal administration as contrasted with the curative dose of 0.275 gm. by this route.

Trypanosoma gambiense.—46 mice infected with *Tr. gambiense* were treated intraperitoneally with doses of the drug ranging from 0.125 to 0.3 gm. per kilo of body weight (Table IV). There were four demonstrable relapses; one mouse treated with 0.25 gm. relapsed on the 11th day, one with 0.15 gm. also on the 11th day, and two with 0.125 gm. on the 14th day. Of the fourteen mice which were given doses of 0.125 and 0.15 gm., three died within the first 15 days from accidental causes, three definitely relapsed, and eight animals survived the 30 day period of cure. Graded doses from 0.175 to 0.3 gm. were given to thirty-two mice, and of this number but one animal treated with 0.25 gm. relapsed. The range of therapeutic activity of the amide of *N*-phenylglycine-*p*-arsonic acid against *Tr. gambiense* infection in mice is definitely greater than against

Tr. brucei infections, since doses of 0.15 gm. failed to cure nagana mice and doses of 0.2 gm. were curative in but five out of thirteen animals.

TABLE IV.

Tr. gambiense Infection of Mice Treated with N-Phenylglycineamide-p-Arsonic Acid 24 Hours after Inoculation. Intraperitoneal Administration.

Dose per kilo.	No. of mice.	No. of intercurrent deaths.	No. of relapses.	No. of cures.
gm.				
0.3	2	1	0	1
0.275	2	0	0	2
0.25	8	0	1	7
0.225	4	1	0	3
0.2	9	0	0	9
0.175	7	1	0	6
0.15	9	2	1	6
0.125	5	1	2	2
Total.....	46			

Trypanosoma equinum.—The results of the treatment of *Tr. equinum* infection in mice are shown in Table V. Twenty-nine mice were treated with intraperitoneal doses of 0.05 to 0.25 gm. per kilo of body weight, with no relapses at 0.2 and 0.25 gm. Doses of 0.1 and 0.15 gm. had a distinct therapeutic effect, and seven of the four-

TABLE V.

Tr. equinum Infection of Mice Treated with N-Phenylglycineamide-p-Arsonic Acid 24 Hours after Inoculation. Intraperitoneal Administration.

Dose per kilo.	No. of mice.	No. of intercurrent deaths.	No. of relapses.	No. of cures.
gm.				
0.25	7	1*	0	6
0.2	7	3†	0	4
0.15	7	1	2	4
0.1	7	0	4	3
0.05	1	0	1	0
Total.....	29			

* Bacterial infection.

† Bacterial infection in one mouse.

teen mice treated with these doses were cured, while in some instances, the life of the uncured animal was considerably longer than that of the untreated control. A dose of 0.05 gm. had no effect whatever on the length of the infection or on the number of parasites in the peripheral blood in the single instance in which this dose was used.

Trypanosoma equiperdum.—In Table VI are given the results of the treatment of thirty-seven dourine mice with this arsenical compound in doses ranging from 0.15 to 0.5 gm. per kilo of body weight. The therapeutic action of the drug on infections in mice produced by our strain of *Tr. equiperdum* was quite irregular. Relapses

TABLE VI.

Tr. equiperdum Infection of Mice Treated with N-Phenylglycineamide-p-Arsonic Acid 24 Hours after Inoculation. Intraperitoneal Administration.

Dose per kilo.	No. of mice.	No. of intercurrent deaths.	No. of relapses.	No. of cures.
gm.				
0.5	4	0	3	1
0.45	4	1	2	1
0.4	4	0	3	1
0.35	6	0	5	1
0.325	2	1	1	0
0.3	6	1	4	1
0.275	2	0	1	1
0.25	4	0	2	2
0.225	2	0	1	1
0.2	2	0	2	0
0.15	1	0	1	0
Total.....	37			

occurred with all doses used irrespective of size, from 4 to 15 days after treatment, and there were no cures below doses of 0.225 gm., although in every instance, even with the lowest doses, the peripheral blood was cleared of trypanosomes for several days and the mice themselves lived from 2 to 8 days longer than the untreated controls. On the other hand, there were some cures at doses of 0.2 to 0.3 gm.

Trypanosoma evansi.—Thirty mice infected with *Tr. evansi* were treated intraperitoneally with the amide of *N*-phenylglycine-*p*-arsonic acid in doses ranging from 0.15 to 0.75 gm. (Table VII).

There were definite cures at all doses above 0.15 gm. with no demonstrable relapse above 0.5 gm. Of the four mice treated with 0.75 gm., one died within the first 30 days after treatment and three were cured. With doses ranging downward from 0.5 gm., relapses occurred irregularly in from 7 to 15 days, but the trypanocidal activity of the drug was shown in all instances even with the lowest doses, since the blood was cleared of parasites for several days following treatment.

TABLE VII.

Tr. evansi Infection of Mice Treated with N-Phenylglycineamide-p-Arsonic Acid 24 Hours after Inoculation. Intraperitoneal Administration.

Dose per kilo.	No. of mice.	No. of intercurrent deaths.	No. of relapses.	No. of cures.
gm.				
0.75	4	1	0	3
0.5	4	1	1	2
0.4	4	0	1	3
0.35	2	1	0	1
0.3	6	1	3	2
0.275	2	0	1	1
0.25	2	0	1	1
0.225	2	1	1	0
0.2	2	0	1	1
0.15	2	0	2	0
Total.....	30			

Treatment of Experimental Trypanosomiasis of Rats.

The therapeutic experiments with rats are likewise grouped in tables according to the various species of trypanosomes employed. As in the case of the mice experiments, the animals listed under "intercurrent deaths" are not included in the number of cured animals, irrespective of the time of occurrence of the death or of the size of the dose administered. Intraperitoneal administration of the drug was employed in every experiment.

Trypanosoma brucei.—Thirty-four rats infected with *Tr. brucei* were treated intraperitoneally with the amide of *N*-phenylglycine-*p*-arsonic acid in doses of from 0.2 to 1.25 gm. per kilo of body weight (Table VIII). There was but one demonstrable relapse at 0.225 gm.

in an animal which showed numerous trypanosomes in its blood 14 days after treatment. With doses of the drug as high as 0.75 gm., rats may show an early and severe intoxication from which recovery is unusual, and such was the case with the rat which was treated with 0.9 gm. and which died on the 4th day. Doses below 0.6 gm. are well borne and are uniformly curative in *Tr. brucei* infections down to 0.25 gm. However, with doses below this level, the therapeutic action of the drug is still marked as shown by the single relapse in five animals treated with 0.2 and 0.225 gm. doses.

TABLE VIII.

Tr. brucei Infection of Rats Treated with N-Phenylglycineamide-p-Arsonic Acid 24 Hours after Inoculation. Intraperitoneal Administration.

Dose per kilo.	No. of rats.	No. of intercurrent deaths.	No. of relapses.	No. of cures.
gm.				
1.25	1	0	0	1
1.0	2	0	0	2
0.9	1	1	0	0
0.75	1	0	0	1
0.6	1	1	0	0
0.5	1	1	0	0
0.4	3	0	0	3
0.375	3	0	0	3
0.35	3	2	0	1
0.325	3	0	0	3
0.3	3	1*	0	2
0.275	3	2*	0	1
0.25	4	0	0	4
0.225	3	0	1	2
0.2	2	1	0	1
Total.....	34			

* Two pregnant females dying 7 and 8 days after treatment.

Trypanosoma gambiense.—Table IX shows the results of the treatment of twenty-five rats infected with *Tr. gambiense* with doses of from 0.1 to 0.3 gm. Doses of 0.2 gm. and above were uniformly curative, no relapses occurring in the fifteen animals treated with 0.2, 0.25, and 0.3 gm. per kilo of body weight. Ten animals were given 0.1 and 0.15 gm. doses, and of this number, there were three relapses and one accidental death. One rat which was treated with

0.15 gm. showed a well developed blood infection on the 18th day and died on the 25th day after treatment. The two rats which relapsed with doses of 0.1 gm. showed trypanosomes on the 10th and 18th days and died on the 25th and 36th days.

TABLE IX.

Tr. gambiense Infection of Rats Treated with N-Phenylglycineamide-p-Arsonic Acid 24 Hours after Inoculation. Intraperitoneal Administration.

Dose per kilo.	No. of rats.	No. of intercurrent deaths.	No. of relapses.	No. of cures.
gm.				
0.3	5	0	0	5
0.25	5	2	0	3
0.2	5	2*	0	3
0.15	5	1	1	3
0.1	5	0	2	3
Total.....	25			

* Animals missing.

Trypanosoma equiperdum.—Eight rats infected with *Tr. equiperdum* were treated with the amide of *N*-phenylglycine-*p*-arsonic acid as shown in Table X. Doses from 0.25 to 0.5 gm. per kilo of body weight were used and no relapse occurred in the higher doses of 0.4 and 0.5 gm. Two rats treated with 0.3 gm. relapsed on the 8th and 16th days and one with 0.25 gm. relapsed on the 7th day. In each case, however, examination of the peripheral blood showed no parasites for several days and the animals all lived longer than the untreated controls.

TABLE X.

Tr. equiperdum Infection of Rats Treated with N-Phenylglycineamide-p-Arsonic Acid 24 Hours after Inoculation. Intraperitoneal Administration.

Dose per kilo.	No. of rats.	No. of intercurrent deaths.	No. of relapses.	No. of cures.
gm.				
0.5	2	0	0	2
0.4	2	0	0	2
0.3	2	0	2	0
0.25	2	1	1	0
Total.....	8			

Trypanosoma evansi.—The results of the treatment of surra infection in rats are shown in Table XI. Nine rats were treated with doses ranging from 0.25 to 0.5 gm. per kilo of body weight, and no relapses were observed with doses above 0.25 gm. Of the three rats which were given 0.25 gm. of the drug, one relapsed with a heavy blood infection on the 14th day after treatment and died on the following day; a second animal which died on the 14th day showed at autopsy a large splenic tumor and was considered a probable relapse; while the third rat in this group, which died 13 days after treatment, is not counted as a cured animal, although no parasites were found in the peripheral blood and the autopsy findings were negative.

TABLE XI.

Tr. evansi Infection of Rats Treated with N-Phenylglycineamide-p-Arsonic Acid 24 Hours after Inoculation. Intraperitoneal Administration.

Dose per kilo.	No. of rats.	No. of intercurrent deaths.	No. of relapses.	No. of cures.
gm.				
0.5	2	1*	0	1
0.4	2	0	0	2
0.3	2	0	0	2
0.25	3	1	2	0
Total.....	9			

* Pregnant female.

Treatment of Experimental Trypanosomiasis of Guinea Pigs.

A number of therapeutic experiments in guinea pigs have been done of which the example given in Table XII is typical. Doses of 0.15, 0.2, and 0.25 gm. per kilo of body weight of the compound given intraperitoneally in a 2 per cent solution were uniformly curative, no relapses having been detected during the period of observation of 3 months. The nagana infection in this particular experiment was severe, as shown by the death of the control animals on the 1st and 3rd days after treatment of the series. Other experiments in which the same and much larger doses of the drug were given showed uniformly curative results and need not be included here.

The therapeutic action of *N*-phenylglycineamide-*p*-arsonic acid upon experimental trypanosomiasis of guinea pigs is in striking accord with that obtained in mice and rats and in marked contrast to that of many trypanocidal drugs which show a greatly lessened therapeutic action in this animal species.

TABLE XII.

Tr. brucei Infection of Guinea Pigs Treated with N-Phenylglycineamide-p-Arsonic Acid 24 Hours after Inoculation. Intraperitoneal Administration.

Dose per kilo.	No. of guinea pigs.	No. of intercurrent deaths.	No. of relapses.	No. of cures.
gm.				
0.25	2	1	0	1
0.2	2	0	0	2
0.15	2	0	0	2
Total.....	6			

DISCUSSION.

As has been shown in the accompanying tables, the curative level of the amide of *N*-phenylglycine-*p*-arsonic acid is quite constant and uniform for most species of trypanosome infections in both mice and rats. Certain exceptions exist which will be referred to later, but in general, with the more virulent strains of organisms and the grade of infection which we have used, single doses of from 0.2 to 0.3 gm. per kilo of body weight given intraperitoneally 24 hours after inoculation are uniformly curative. The parasites promptly disappear from the peripheral blood within 24 hours following treatment at this level of dosage and barring death from accidental causes, the animals survive the observation period of 1 or 2 months with no return of the infection. For mice the minimum lethal dose of this drug given intraperitoneally has been placed at 2 to 2.25 gm. per kilo of body weight, and since the curative dose may be considered as 0.275 gm., we have a curative ratio of 1:8. Similarly the minimum lethal dose of the drug for rats has been estimated at 0.75 gm. per kilo of body weight, and the curative ratio for rats, therefore, is 1:3.

In addition, the range of therapeutic activity, as indicated by the

dose that will temporarily clear the blood of trypanosomes as well as effect a certain proportion of cures, varies somewhat with the species of organism. Generally speaking, with the more virulent and more rapidly fatal trypanosomes, the clearing dose closely approaches the curative dose. Thus, with *Tr. brucei* infections in mice, 0.2, 0.225, and 0.25 gm. were usually clearing doses; while with *Tr. gambiense* and *Tr. equinum*, 0.1, 0.125, and 0.15 gm. cleared the blood. On the other hand, with less virulent strains, such as *Tr. equiperdum* and *Tr. evansi*, the clearing dose is considerably below the curative level. Thus, with these strains, doses of 0.15 to 0.25 gm. cleared the blood of trypanosomes for several days, although the curative dose for both strains must be placed above 0.5 gm. The range of therapeutic activity as expressed in the size of the clearing dose is practically the same in the rat as in the mouse infections.

Different species of trypanosomes vary considerably in their resistance to therapeutic agents, and in this respect, the therapeutic action of this compound is not maintained at the constant curative level of 0.2 to 0.3 gm. per kilo of body weight. Moreover, the animal species employed may to some extent influence the control of certain infections. The curative range of 0.2 to 0.3 gm. is quite constant for *Tr. brucei*, *Tr. gambiense*, and *Tr. equinum* infections in mice and *Tr. brucei* and *Tr. gambiense* infections in rats, but with *Tr. equiperdum* and *Tr. evansi* infections in mice, the dose required to effect a cure is considerably higher. The highest dose used, 0.5 gm., failed to cure all the mice infected with *Tr. equiperdum*, although there were cures at doses as low as 0.225 and 0.25 gm. With infections of *Tr. evansi*, mice treated with 0.75 gm. did not relapse, but below this level, cures were uncertain. Both these infections, however, were more easily handled in rats than in mice, since there were no relapses with doses of 0.4 gm. in *Tr. equiperdum* and none with 0.3 gm. in *Tr. evansi* infections.

The lower level of therapeutic action of the drug is quite sharp with the more virulent species of trypanosomes. Relapses with *Tr. brucei* infections in mice occurred with 0.25 gm. (intraperitoneal administration) and increased in number with lower doses, while with *Tr. gambiense* and *Tr. equinum* infections in mice, relapses began with 0.15 gm., with the single exception of one *Tr. gambiense* mouse which

relapsed with a 0.25 gm. dose. It is possible that this relapse might have been due to a leakage of the drug at the time of treatment, since this was the only observed relapse in twenty-eight mice treated with doses of from 0.175 to 0.25 gm. The majority of relapses with these infections occurred from 5 to 12 days after treatment. The level of relapse occurrence with *Tr. brucei* and *Tr. gambiense* infections in rats is quite comparable with that observed in mice, while the time of occurrence was from 14 to 25 days after treatment. With the less virulent organisms which were used, *Tr. equiperdum* and *Tr. evansi*, the therapeutic action of the amide of *N*-phenylglycine-*p*-arsonic acid is much less clear-cut and regular. Relapses occurred in mice with doses of from 0.15 to 0.5 gm. and in general from 7 to 15 days after treatment. The therapeutic action in rats, however, is more constant and more sharply limited, for with *Tr. equiperdum* infections, there were no relapses above 0.3 gm. and with *Tr. evansi* none above 0.25 gm. The relapses with both these species in rats occurred from 8 to 16 days after treatment. It will be recalled that in all the experiments with both mice and rats, and irrespective of the species of trypanosome employed, treatment was given on the day following the inoculation of the animals and that the therapeutic results described above apply to the treatment of a 24 hour infection. The average curative level, the clearing dose, and the number and time of relapses would undoubtedly be affected with any considerable change in the time of treatment.

The therapeutic results in the treatment of experimental trypanosomiasis of guinea pigs, as shown by the low curative dose of 0.15 gm. per kilo of body weight in a severe infection, are even better than those obtained in the treatment of mice and rats and are in striking contrast to the action of certain other trypanocidal agents. The curative ratio expressed in fractions of the minimum lethal dose is 1:10, since the lethal dose for guinea pigs is 1.5 gm. per kilo of body weight given intraperitoneally or subcutaneously.

The rapidity of the trypanocidal action of the amide of *N*-phenylglycine-*p*-arsonic acid on trypanosomiasis of mice, rats, and guinea pigs is quite marked. That is, with curative doses, the drug becomes quickly available after its administration and remains in an active biological state long enough to accomplish its trypanocidal action, for

in 18 to 24 hours after the treatment of a 24 hour infection, the peripheral blood is free of parasites. Moreover, with subcurative but clearing doses, a similar speed of trypanocidal action is shown. This speed of trypanocidal activity is operative in both mice and rats, but in the case of our strains of *Tr. equiperdum* and *Tr. evansi*, the action is apparently somewhat more drastic in rats. In mice the therapeutic action extends over a wide range of doses and cures are irregularly distributed among the low as well as among the high limits of this range, while treatment of the same infections in rats reveals a more sharply limited therapeutic action. It is of interest in this connection to compare the results of the administration of the drug by different routes in the treatment of *Tr. brucei* infections of mice. By all three routes, intraperitoneal, intravenous, and subcutaneous, the sharpness of therapeutic action is maintained within the curative range and apparently the drug is as available in an active biological state by subcutaneous as by intraperitoneal or intravenous administration. The therapeutic experiments in guinea pigs are too few to admit final analysis, but apparently the rapidity and sharpness of action of the drug likewise obtain in this animal species.

One other point regarding the action of the drug should be mentioned and this is in connection with the general condition of the treated animals. Practically without exception, all the cured animals showed a marked improvement manifested by a gain in weight and by the healthy appearance of their coats which dated from the time of treatment and continued during the period of observation. The cured animals were usually in a better condition than those of the original stock. Rats which were treated with high doses approaching a lethal level and which developed toxic manifestations did not recover, but aside from this evidence of drug toxicity, the treated animals were uniformly in excellent state. The negative autopsy findings in animals that were treated with therapeutic doses of the amide of *N*-phenylglycine-*p*-arsonic acid furnish additional corroborative evidence of the non-injurious action of such doses to the host.

CONCLUSIONS.

N-Phenylglycineamide-*p*-arsonic acid is an agent of marked therapeutic action in the treatment of experimental trypanosomiasis of mice, rats, and guinea pigs. It possesses an average curative range of from 0.2 to 0.3 gm. per kilo of body weight of the sodium salt against a 24 hour infection in mice and rats produced by several species of pathogenic trypanosomes. Since the lethal dose for mice is from 2 to 2.25 gm. and for rats 0.75 gm. per kilo of body weight, we have curative ratios of 1:8 and 1:3 respectively. The curative dose for guinea pigs is 0.15 gm. per kilo of body weight, thus giving a curative ratio of 1:10. The trypanocidal activity of this compound is relatively rapid in all three animal species, for the peripheral blood is cleared of organisms within 24 hours after its administration, and in addition, the lower limits of the curative range are comparatively sharply defined. Intraperitoneal, intravenous, and subcutaneous routes of administration for all practical purposes may be considered equally efficacious in *Tr. brucei* infections of mice both as regards the speed of action of the drug and the average curative range. The administration of the drug in therapeutic amounts in all three animal species is not followed by manifestations of organic or functional injury, but, on the contrary, the general physical condition of the treated animals shows an immediate and continued marked improvement.

The therapeutic activity in trypanosomiasis of mice, rats, and guinea pigs as evidenced by the relative speed and sharpness of action, together with the curative ratio as expressed in fractions of the minimum lethal dose, and the absence of organic injury or functional disturbance following therapeutic doses are significant and characteristic features of the amide of *N*-phenylglycine-*p*-arsonic acid.

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CHEMOTHERAPY OF TRYPANOSOME AND SPIROCHETE INFECTIONS.

Biological Series. III.

The Therapeutic Action of N-Phenylglycineamide-*p*-Arsonic Acid in Experimental Trypanosomiasis of Rabbits.

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Plates 17 to 37.

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Experimental trypanosomiasis of rabbits, as has been pointed out, presents many points of similarity with naturally acquired trypanosomiasis of human beings and animals, and since the treatment offers an opportunity of obtaining fundamental information which may be of practical importance in the treatment of trypanosomal disease as it exists in nature, we have endeavored to emphasize such fundamental points in the description of the therapeutic results with the amide of *N*-phenylglycine-*p*-arsonic acid reported in this paper.

EXPERIMENTAL.

Condition of Experiments.—The majority of therapeutic experiments were carried out with strains of *Tr. brucei* and *Tr. gambiense* and a smaller number of experiments with *Tr. equiperdum*, *Tr. equinum*, and *Tr. evansi*. The infection produced by all five strains was uniformly fatal in untreated rabbits. The infecting virus in all experiments was, with one exception, obtained from stock guinea pigs in which the trypanosome strains were continuously carried, thus conforming to the principle of the influence of the animal species upon the type of infection which has been discussed in a previous paper.[1] In addition,

[1] Pearce, L., and Brown, W. H., *J. Exp. Med.*, 1918, xxviii, 109.

in order to secure regularity of incubation as well as uniformity of character and termination of the infection in as far as this was possible, blood was taken from stock guinea pigs which showed a well developed infection and the inoculating doses of the blood suspension were given intravenously. With *Tr. brucei*, *Tr. equiperdum*, and *Tr. evansi*, 1 cc. per kilo of body weight of a + suspension was inoculated, while with *Tr. gambiense* and *Tr. equinum*, 1 cc. per kilo of a ++ to +++ suspension was used. Under these conditions of experimental procedure, rabbits infected with *Tr. brucei* showed initial signs of the disease in from 5 to 7 days and the untreated animals survived from 1 to 3 months, although the majority of controls died within the first 5 weeks, while the first signs of the infection with *Tr. gambiense* were noted 7 to 15 days after inoculation and untreated animals survived on an average of 8 weeks. The clinical course of the infection produced in rabbits by our strains of *Tr. equinum*, *Tr. evansi*, and *Tr. equiperdum* is, on the whole, comparable with that caused by *Tr. brucei*.

Rabbits thus infected were treated with the sodium salt of the amide of *N*-phenylglycine-*p*-arsonic acid at a time when the infection was well established, usually about 10 to 15 days after inoculation with *Tr. brucei* and at a somewhat longer period of time with the other strains of trypanosomes, when well marked clinical signs of the disease together with loss of appetite, weakness, and a concomitant loss of weight or even emaciation were present. In a certain number of cases, particularly in the treatment of relapses, rabbits showing a more advanced or even a chronic stage of trypanosomiasis were used.

With regard to the matter of dosage, it is important to recognize that since individual rabbits vary considerably in their reaction to trypanosomal infections as shown by the character and degree of the clinical signs, we have endeavored to utilize this factor by placing some of the animals most affected at the crucial dose levels, that is, within the curative range. The dose of the drug for each animal was calculated in grams per kilo of body weight, weighed on a balance, dissolved in sterile distilled water, and injected by a syringe into the marginal ear vein except in the few instances in which the drug was administered intramuscularly, subcutaneously, or *per os* through a stomach tube. The concentration of the drug solutions varied from 5 to 50 per cent, although in the majority of experiments a 5 per cent solution was used.

The immediate and ultimate therapeutic effect of the drug in the treatment of experimental trypanosomiasis in rabbits was closely observed throughout a period of at least 3 months and in many instances for a longer period of time, although the majority of relapses after non-curative doses of the amide of *N*-phenylglycine-*p*-arsonic acid occurred in our experiments within 2 to 5 weeks after treatment.

Determination of a Single Dose Curative Range.

Our initial experiments with the amide of *N*-phenylglycine-*p*-arsonic acid were largely concerned with the determination of the range of therapeutic action and a study of the effect of the drug within this range rather than the determination of a single curative dose or an attempt to ascertain the ideal method of treating and of curing experimental trypanosomiasis. Although the treatment of trypanosomiasis in this animal species resolves itself for the most part into the treatment of individuals, we have grouped together in Table I the results of a number of experiments which have certain features in common. In these experiments, thirty-four rabbits infected with *Tr. brucei* were treated 8 to 15 days after inoculation, at a time when all showed well marked clinical signs and symptoms of the disease. Some of the control animals died from 20 to 36 days after treatment, while others which showed extremely advanced signs at the end of 42 days were used for therapeutic purposes. The single doses of the drug employed in the treatment of these rabbits, as is shown in the table, ranged from 0.75 to 0.1 gm. per kilo of body weight and were administered intravenously in a 5 per cent solution.

There were no evidences of drug intoxication after treatment with single doses of this range and the clinical signs of trypanosomiasis were quickly influenced. Usually the acute manifestations of the infection, such as edema, capillary dilatation and congestion, and swelling of the base of the ears, eyelids, prepuce, and testicles, largely subsided within 24 to 48 hours, while the reduction of the more indurated and advanced lesions required from 2 to 4 days. The immediate clinical results of the treatment are illustrated in Figs. 1 to 7. Apart from the outward signs of the disease, the animal's general condition improved markedly following the injection of the drug as shown by

a rapid return of appetite and an increase in weight, so that within a few days emaciation had given place to a more or less well nourished condition, while in the succeeding weeks, a normal increase in weight occurred.

The final results in this series are given in Table I. There were no relapses among the twelve rabbits treated with doses of both 0.35 and 0.25 gm. per kilo of body weight, while with the intermediate dose of

TABLE I.

Results Obtained in the Treatment of Rabbits Infected with Tr. brucei with Single Doses of N-Phenylglycineamide-p-Arsonic Acid, Administered Intravenously.

Dose per kilo.	No. of rabbits.	No. of intercurrent deaths.	No. of relapses.	No. of cures.	Percentage of cures.
gm.					
0.75	1	0	0	1	100
0.4	2	0	0	2	100
0.35	6	2*	0	6	100
0.3	7	0	1	6	86
0.25	6	3†	0	6	100
0.2	10	1‡	3	7	70
0.1	2	0	2	0	0
Total.....	34				

* One was killed 71 days after treatment; extensive ringworm. One died 73 days after treatment; middle ear abscess.

† One died the day before the series was discarded; hemorrhagic septicemia. One died 71 days after treatment; acute pneumonia. One was killed 71 days after treatment; extensive ringworm.

‡ Killed 77 days after treatment; bacterial infection.

0.3 gm. per kilo, there was one relapse out of seven animals, or 86 per cent of cures. Furthermore, with a lower dose of 0.2 gm. seven out of ten rabbits were cured (70 per cent), and finally, the two rabbits treated with the very low dose of 0.1 gm. relapsed, although the marked therapeutic activity of the drug at this level is shown by the fact that with rabbits so treated, regression and healing of the lesions took place very rapidly and the relapses did not occur until 11 and 21 days after treatment. There were six incidental deaths among the thirty-four rabbits included in this table, but since they occurred over 10 weeks after treatment and were in no way associated with evidences

of trypanosomiasis, we have not excluded them from the number of cured animals.

The outstanding feature of the treatment of an acute, progressive, and well established *Tr. brucei* infection in rabbits with single doses of the amide of *N*-phenylglycine-*p*-arsonic acid as shown in Table I is a well marked sharply limited curative range of therapeutic action with doses varying from 0.2 to 0.35 gm. per kilo, and that within these limits, the percentage of cures is, on the average, high. Thus, there were twenty-nine rabbits treated with doses of 0.2, 0.25, 0.3, and 0.35 gm. per kilo of body weight and of these four relapsed and twenty-five, or 86.2 per cent, were cured. In addition, both upper and lower limits of action of the curative range are sharply marked—with doses of 0.2 gm., there were 70 per cent of cures and with the lower dose of 0.1 gm., there were no cures although this amount of the drug possessed a marked therapeutic effect, while, on the other hand, doses of 0.35 gm. or higher were uniformly curative. If we consider that the minimum lethal dose of the drug for rabbits is from 0.75 to 0.9 gm. per kilo of body weight when given intravenously and that the curative range of single doses is from 0.25 to 0.35 gm., we have a curative ratio of from one-third to one-half as expressed in fractions of the minimum lethal dose.

Much the same range of curative action is operative in rabbit trypanosomiasis produced by *Tr. gambiense* as is shown in Table II. In this series, eighteen rabbits were treated intravenously with doses of the amide of *N*-phenylglycine-*p*-arsonic acid of 0.35 to 0.15 gm. per kilo of body weight. There was a very noticeable regression of the signs of the infection on the 1st and 2nd days after treatment, and no detectable signs remaining on the 5th day, as may be seen in Figs. 8 and 9. Two out of the three rabbits treated with 0.15 gm. per kilo relapsed 20 and 33 days after treatment, and there was one relapse at 0.25 gm. 26 days after treatment, but the eleven rabbits treated with the other three doses of 0.2, 0.3, and 0.35 gm. per kilo were cured. Thus the curative range of the compound in *Tr. gambiense* infections of rabbits may be considered to be from 0.2 to 0.3 gm. per kilo when administered as single doses intravenously—or from one-fourth to one-third of the minimal lethal dose.

Our experience with the effect of this compound upon rabbit trypanosomiasis produced by other species of trypanosomes is limited

so that we are unable to draw final conclusions as to the ultimate curative range of the drug in these particular infections. At the time the experiments were done, the infection produced in these animals by our strain of *Tr. equinum* was unusually severe as was also the case in certain mouse experiments, and in addition, the upper range of the doses used was apparently too low. However, single doses as small as 0.2 gm. per kilo of body weight caused a complete disappearance of the acute signs of the disease in 3 days, although relapses eventually occurred with the doses 0.15, 0.2, 0.25, and 0.3 gm. per kilo. It was obvious that larger doses should have been used or a different system of treatment followed to effect a permanent cure in an infection as

TABLE II.

Results Obtained in the Treatment of Rabbits Infected with Tr. gambiense with Single Doses of N-Phenylglycineamide-p-Arsonic Acid, Administered Intravenously.

Dose per kilo.	No. of rabbits.	No. of intercurrent deaths.	No. of relapses.	No. of cures.	Percentage of cures.
gm.					
0.35	3	0	0	3	100
0.3	4	0	0	4	100
0.25	3	1*	1	1	50
0.2	5	1†	0	4	100
0.15	3	2‡	2‡	1	33⅓
Total.....	18				

* Died 6 days after treatment; bacterial infection.

† Died 20 days after treatment; gastroenteritis.

‡ Relapse plus bacterial infection 20 and 33 days after treatment.

severe as was the case in this particular experiment, since the control rabbit died the day after the animals in the series were treated and relapses occurred with such promptness and severity.

Two rabbits heavily infected with *Tr. equiperdum* which were treated with single doses of 0.5 and 0.3 gm. of the amide of *N*-phenylglycine-*p*-arsonic acid per kilo of body weight, 36 days after inoculation, at a time when the disease was advanced, showed a prompt recovery from the clinical signs as is illustrated by Figs. 12 and 13. Both rabbits subsequently relapsed on the 47th and 57th days after treatment but were successfully retreated with repeated doses of the drug with no ultimate recurrence of the infection.

The therapeutic effect of the compound upon *Tr. evansi* in rabbits was studied in but one animal. The infection was well established on the day of treatment, 36 days after inoculation, as is shown in Fig. 14, when 0.3 gm. per kilo was administered intravenously. There was very little change in the clinical signs during the first 48 hours but on the following days, the improvement was marked (Figs. 15 and 16). Thick crusts about the nose and upper lips had shelled off in 10 days time and by the end of the 3rd week, the skin in these areas was healthy in appearance and the hair had begun to return (Fig. 17). There was no relapse and the rabbit was in excellent condition when it was discarded 113 days after treatment.

Summing up the results of the treatment of trypanosomiasis with single doses of the drug, we may say that with infections of average severity and duration produced by *Tr. brucei* and *Tr. gambiense*, the drug exercises a prompt and lasting therapeutic action in doses of from 0.2 to 0.35 gm. per kilo of body weight, and, furthermore, a marked therapeutic effect of some duration in doses of 0.1 and 0.15 gm. per kilo of body weight. Although the experimental evidence with regard to *Tr. evansi* infection in rabbits is insufficient for final conclusions, it is probable that the same range of curative action would apply to such rabbit infections as are produced by the particular strain of surra with which we have worked. On the other hand, single doses of the compound which fall within this curative range failed to cure *Tr. equinum* and *Tr. equiperdum* infections in rabbits. However, it should be pointed out that in both these experiments, especially with *Tr. equinum*, the disease was extremely marked and more advanced at the time of treatment than was the case with the other three infections, and it is possible that with more comparable experimental conditions, a certain proportion of cures could be obtained with single doses falling within the curative range of 0.2 to 0.35 gm. per kilo of body weight.

In addition to the above experiments, a series of twelve rabbits with a very severe *Tr. brucei* infection of 23 days duration was treated intravenously with large single doses in order to ascertain the reaction of extremely ill animals to the drug as compared with that of normal animals. The dose of 0.9 gm. per kilo of body weight caused toxic symptoms in the three rabbits so treated, with death on the 2nd and

3rd days, while a similar result followed the dose of 0.75 gm. in two out of three rabbits; with 0.6 gm., only one rabbit of the three became toxic and eventually recovered, and the dose of 0.5 gm. caused no toxic disturbances whatever. From the above results, it is evident that the tolerance of very ill rabbits suffering from a severe acute trypanosomiasis infection compares very favorably with that of normal animals to single large doses of *N*-phenylglycineamide-*p*-arsonic acid administered intravenously.

Treatment of Initial Infections with Repeated Doses.

In many respects, the treatment of rabbit trypanosomiasis with repeated small doses of the amide of *N*-phenylglycine-*p*-arsonic acid more nearly meets conditions as they are found in nature, and in addition the control and ultimate cure of severe and long standing infections which might not yield to a single dose treatment may often be successfully accomplished by this system of therapeutic procedure. This is due in part to the fact that with the debilitated and weak condition of the animal host, it may not be wise to give a single dose of sufficient size and probably in part also to the chronic and indurated nature of the lesions present, the penetration of which may be difficult and may require considerable time. By observing the action of single small doses of the drug, we form an idea of their effect and duration. Obviously, the repetition of a dose should occur before the effect of the previous dose has ceased, and we consider the proper spacing of doses as important a factor in this connection as the number and size of the doses. Finally, all these factors must be largely determined by the physical state of the diseased animal as well as by the age, severity, and type of the infection.

We have treated a number of rabbits infected with *Tr. brucei* with three doses of the compound ranging from 0.2 to 0.05 gm. per kilo repeated at various intervals of time, of which the following are typical examples. Two rabbits infected with *Tr. brucei* were treated 11 days after inoculation, with three doses of 0.2 gm. per kilo repeated at 2 day intervals. The disease was well established with marked typical clinical signs at the time of treatment. Both animals recovered promptly with no signs of drug intoxication and no return of the

infection. Two other rabbits in the same series treated with three doses of 0.15 gm. at 2 day intervals likewise showed a prompt and complete disappearance of the clinical signs of the infection but one animal eventually relapsed 30 days later. Another animal which was given three doses of 0.1 gm. at 2 day intervals remained free from any signs of the infection for 186 days at which time it was discarded as cured. The last rabbit in this series which was given three doses of 0.05 gm. relapsed 13 days after treatment had been begun.

A modification of the spacing of repeated small doses was followed in the case of another animal which was treated 14 days after inoculation with *Tr. brucei*; the initial dose was 0.1 gm. per kilo. 8 days after treatment, the return of clinical signs of trypanosomiasis was observed about the head and nose, and the dose of 0.1 gm. per kilo was repeated 2 days later. The clinical signs cleared up promptly within 4 days and the animal remained in good condition without relapse during the period of observation following treatment.

In the experiments just cited, the infections were of an acute, rapidly progressive type. The effect of treatment with repeated doses of this arsenical upon a slowly developing severe infection of long standing is illustrated in Figs. 18 to 23. In this instance, the rabbit was inoculated with *Tr. brucei* in the usual manner but the infection was relatively slow in developing and treatment was not begun until 48 days after inoculation, when three doses of 0.2 gm. were given intravenously on succeeding days. At this time, the animal was extremely weak and emaciated, the ears, eyelids, face, nose, and external genitalia were swollen and indurated, and in addition, there were a mucopurulent exudate and scab formation about the eyes and nose (Fig. 18). An immediate improvement in the clinical manifestations of the disease was seen after treatment, although there was a loss of weight for the first 5 days, but by the 7th day this had largely been regained. Within the week following treatment, the scabs about the ears and around the eyes and end of the nose and lips had scaled off almost entirely, leaving a fairly healthy looking skin, the indurated swellings about the head and genitalia had greatly subsided, and the general condition of the rabbit had improved remarkably as is shown in Figs. 19 and 20. By the end of 3 weeks, the change in the appearance of the rabbit as contrasted with its condition on the day of treatment was

quite striking (Fig. 22), and in 6 weeks, a small bald patch at the base of the left ear was the only residual sign of a previous lesion (Fig. 23). The recovery from a severe and long standing infection under the system of three small doses of 0.2 gm. per kilo repeated at daily intervals was complete and lasting, for there was no subsequent relapse during the period of observation of 195 days.

The treatment of initial infection with *Tr. brucei* in rabbits, therefore, may be successfully accomplished by a system of repeated small doses provided that the repetitions occur well within the time of duration of action of the previous dose. The value of this system of therapy is especially marked in instances of greatly weakened animals or in those in which chronic indurated lesions are pronounced.

Treatment of Relapses with Single Doses.

The successful treatment of trypanosomiasis relapses in rabbits, many of which were intentionally produced as test objects, is based largely upon the severity and duration of the relapse and the general physical condition of the rabbit. While the factor of fastness of trypanosomes due to previous treatment unquestionably plays a part in the blood infections of mice and rats and must be considered to a certain extent in the tissue infection of rabbits, it is our opinion that the severity and duration of the relapse as manifested by the signs and symptoms of the actual infection, together with the general physical state of the rabbit host, play a more important rôle. It is obvious that the treatment of a relapse is almost wholly the treatment of individuals, and hence no rules of procedure can be laid down; but because of the inherent condition imposed by the infection, we believe that in most cases relapses can best be handled by a system of repeated doses, the size and time of repetition of which are necessarily determined by the extent, severity, and age of the disease together with the physical condition of the animal host. However, if a single large dose is used, it must of necessity cause no serious degree of intoxication or pathological injury, and yet at the same time, it must be of sufficient strength to penetrate diseased tissues of more or less induration and in like manner to act as a trypanocidal agent for a sufficient length of time. A number of relapses in rabbits have

been treated with single doses of this arsenical, of which the following will serve as examples.

The appearance of a *Tr. brucei* relapse of a week's duration at the time of retreatment is shown in Fig. 24. This rabbit was originally treated intravenously with three doses of 0.05 gm. per kilo of the drug which had served to clear up all outward manifestations of the disease, but 13 days later, definite signs of a relapse were noted. The infection was allowed to progress for a week at which time the clinical signs had rapidly increased both in degree and extent. The general physical condition of the rabbit was good. The entire left side of the face was greatly swollen and the left eye completely closed; the upper eyelid was very puffy, and protruding and escaping from between the margins of both lids, which were reddened with considerable loss of hair, was a slightly purulent exudate. The right upper eyelid, right cheek, and upper lip were also slightly swollen. Both ears, especially the left, which drooped markedly, were swollen, inflamed, and edematous and the right testicle was somewhat indurated. The dose selected for retreatment in this instance was 0.5 gm. per kilo of the amide of *N*-phenylglycine-*p*-arsonic acid given intravenously; it was followed by no toxic symptoms whatever. There was a most marked reduction of the clinical signs on the 2nd day; in a week's time the appearance of the rabbit was quite normal as is shown in Fig. 25, and this continued with no return of the infection during the period of observation of 166 days.

Another nagana rabbit which had been treated intravenously with the single dose of 0.29 gm. of the drug per kilo of body weight relapsed 26 days after treatment and the signs of the disease increased extremely rapidly for the succeeding 8 days. A single dose of 0.4 gm. per kilo of the drug was given intravenously. This was followed by a marked immediate improvement in the clinical signs of the infection and in 10 days the animal had regained its original weight and its appearance was entirely normal. There was no further relapse during the 149 day period of observation.

In addition to the treatment of rabbits infected with *Tr. brucei* which had relapsed after the initial treatment with the amide of *N*-phenylglycine-*p*-arsonic acid, a number of animals were treated that had relapsed from initial treatment with single doses of arsphenamine

and neoarsphenamine (German salvarsan and neosalvarsan), ranging from 0.005 to 0.02 gm. per kilo of body weight. For purposes of comparison, the original series which included these rabbits was conducted under the same conditions as were the parallel experiments in which the amide of *N*-phenylglycine-*p*-arsonic acid was used, and the infection was of the same acute nature with marked clinical signs at the time of treatment. The animals which relapsed after the treatment with arsphenamine and neoarsphenamine were held until the infection had progressed to an extreme degree with conspicuous clinical signs and a generally poor physical state of the animals; they were then treated with single doses of *N*-phenylglycineamide-*p*-arsonic acid. Five animals were given 0.5 and eight were given 0.4 gm. per kilo of body weight and all promptly recovered from the signs of the infection, with no evidences of drug intoxication and with a very noticeable and rapid improvement in their physical condition. Three rabbits in this group were killed 34, 47, and 56 days after treatment because of extensive ringworm or middle ear disease, but at autopsy no evidence of trypanosomiasis was found and none of the remaining ten rabbits suffered a relapse from the retreatment with *N*-phenylglycineamide-*p*-arsonic acid.

From the experimental evidence, therefore, it is seen that rabbits infected with our strain of *Tr. brucei* which have relapsed with severe clinical signs after insufficient treatment with the amide of *N*-phenylglycine-*p*-arsonic acid, arsphenamine, and neoarsphenamine may be permanently cured with single doses of 0.4 and 0.5 gm. per kilo of body weight of the amide of *N*-phenylglycine-*p*-arsonic acid.

Treatment of Relapses with Repeated Doses.

In the treatment of relapses with repeated doses, the chief factors of importance to be considered are first, the severity and type of the infection and the general physical condition of the rabbit. If the relapse is long standing with chronic indurated lesions, a single dose of the drug may not penetrate the lesions sufficiently or operate over the necessary length of time required for resolution of such lesions. Moreover, as is frequently the case with this type of chronic relapse, the physical state of the rabbit may be very poor and a single large dose

of the drug might under these conditions prove somewhat dangerous. Hence, from a consideration of these factors, it would seem that a system of small repeated doses, properly spaced, is the most rational system of treating chronic trypanosomal relapses of rabbits. The size of the initial dose or doses should be large enough to exert some influence upon the infection and its lesions, and subsequent doses should be given before the effect of the previous dose has completely worn off. Consequently a knowledge of the general effect and duration of action of various sized doses of this arsenical, as well as accurate and frequent clinical observations of the rabbits under treatment, is essential to a satisfactory outcome with this system of therapy. In this connection, it is important to bear in mind that rabbits as well as other animal species exhibit a high degree of tolerance for the drug as has been shown elsewhere,[2] and this attribute which exists in rabbits infected with trypanosomiasis as well as in non-infected animals may be used to great advantage in the treatment of trypanosomiasis, both of the initial infections and of the relapses. Our experience in handling relapses with repeated doses of this drug has been extremely varied because we have purposely endeavored to ascertain what might be accomplished with various sized doses given at various intervals of time under the many different conditions imposed by the infection rather than an attempt to effect a cure in every case. We have selected certain typical protocols which will illustrate some of the essential points in question.

Fig. 26 shows the appearance of an 8 day relapse in a rabbit which had been treated 34 days before with 0.02 gm. of arsphenamine (German salvarsan) per kilo of body weight. The signs of the relapse increased rapidly and at the time of retreatment with the amide of *N*-phenylglycine-*p*-arsonic acid, the rabbit was not in a particularly good general condition. The face was markedly swollen and indurated, both eyes were closed, the base of both ears was involved in the inflammatory process, both testicles were swollen and indurated, and the prepuce was extremely swollen and congested. Three doses of 0.15 gm. of the amide of *N*-phenylglycine-*p*-arsonic acid were given intravenously on successive days. There were no evidences of drug intoxication whatever, the general condition improved immediately, and

[2] Brown, W. H., and Pearce, L., *J. Exp. Med.*, 1919, xxx, 417.

the clearing up of the clinical signs progressed rapidly as is seen in Fig. 27, which shows the appearance 6 days after treatment had been begun. At this time there remained only a slight swelling and induration of the face and a slight degree of resistance of normal size testicles. These residual signs had completely disappeared on the following day. Unfortunately no opinion of the ultimate therapeutic success of the treatment was possible in this particular case as the animal succumbed to a bacterial infection 24 days after treatment.

Fig. 28 illustrates the appearance of a relapse of 15 days duration in a rabbit that had been treated 49 days previously with 0.02 gm. of neoarsphenamine (German neosalvarsan) per kilo of body weight. There was a very marked swelling of the face and the base of the ears, particularly of the left, an indurated swelling of the eyelids with crust formation, a purulent exudate, loss of hair and complete closure of both eyes, a mucoserous nasal discharge, and a moderate involvement of the genitalia. In addition, the animal was very thin and weak. A dose of 0.5 gm. of the amide of *N*-phenylglycine-*p*-arsonic acid per kilo was given intravenously and was followed by a marked general improvement and gradual disappearance of the signs of the infection with an accompanying gain in weight, although the rabbit still remained weak and middle ear disease developed. 2 weeks after the first dose of the drug had been given, a second dose of 0.5 gm. per kilo was given intravenously with no evidences of drug intoxication but with a decided improvement in the animal's general condition, although the local condition in the right middle ear continued to progress, causing a marked twisting of the head to the right. In the course of recovery from the trypanosomal infection, a large portion of diseased skin over the bridge of the nose and about the eyes gradually desquamated, leaving a healthy looking but bare surface denuded of hair. The growth of new hair in these areas was quite remarkable as is shown in Fig. 29. There was no later evidence of trypanosomiasis observed in this rabbit either clinically or at autopsy, and despite the middle ear abscess which was eventually the cause of death 59 days after the treatment of the relapse, the general physical condition remained excellent.

We have selected two examples of the treatment of nagana relapses of a more prolonged and chronic type which are generally considered to be much more difficult of permanent cure than the types previously

considered in this section. The first of these rabbits was originally treated intravenously with 0.2 gm. of the amide of *N*-phenylglycine-*p*-arsonic acid per kilo of body weight which sufficed to clear up all signs of the infection. However, 27 days later, the diagnosis of a probable relapse was made because of signs about the eyelids, and although at the end of a week's time, the clinical signs of trypanosomiasis were outspoken, the relapse was allowed to progress and the rabbit was not treated until 22 days after the first signs had been noted. The appearance of the head of the rabbit on the day of retreatment is shown in Fig. 30. There was a marked edematous swelling of the face involving the cheeks and eyelids and the eyes were almost closed; both upper lips were extremely swollen and congested with some loss of hair and scab formation; the lower portions of the ears were considerably indurated and very hot to the touch and at the base of the right ear was a necrotic area the size of a silver half dollar. The prepuce was edematous, congested, and somewhat swollen and both testicles although not enlarged were indurated. The general physical condition of the rabbit was fairly good. Two doses of 0.4 gm. of the amide of *N*-phenylglycine-*p*-arsonic acid given a week apart caused a marked improvement in the rabbit's condition as shown in Figs. 31 and 32, but 3 days after the second dose, there was a swelling of the left cheek and both left eyelids, with a slight mucopurulent discharge from the eye. The succeeding four doses (0.6, 0.5, 0.5, and 0.75 gm. per kilo) given over a period of 28 days were eventually effective in clearing up the clinical signs (Figs. 33 to 35), but a relapse ultimately occurred during the summer. Since the total amount of the drug administered was in our experience sufficiently large to cure a relapse of this duration and type, it would seem that the failure was due to the incorrect spacing of the doses which were too far apart. It is probable that if the first three doses had been given on successive or even on alternate days, a permanent cure would have been obtained.

As a contrast to the system of treatment of a relapse pursued in the preceding experiment which failed to effect a permanent cure, the following instance is given of the successful treatment of a chronic and very advanced relapse, a type of infection generally considered extremely difficult of cure. A rabbit infected with *Tr. brucei*, which had been treated in the spring, relapsed during the summer, and at the time of retreatment, about 3 months later, presented an extreme

picture of a long standing chronic relapse (Figs. 36 and 37). A large portion of the face including the area around both eyes and the bridge of the nose was practically devoid of hair. The skin had lost its elasticity, was quite firm, resistant, and indurated, and there were numerous small scabs over the surface, especially around the right eye. The base of the right ear was also swollen and indurated and there was a small raw thickened area on the right hind foot covered with reddish yellow scabs. The prepuce was markedly swollen and quite firm; both testicles although not enlarged were very firm and indurated. The rabbit was weak but in a fairly good state of nutrition. Treatment was begun with the injection of 0.75 gm. of the drug per kilo of body weight and repeated on the 4th and 6th days—a dose which we believed was unnecessarily large, yet the reaction and effect of which we wished to determine in this particular type of relapse.

The therapeutic effect of the drug upon the clinical lesions was most marked as is shown in Figs. 38 to 40. There was no loss of weight, the rabbit's general condition was very noticeably improved, and, moreover, there was no indication of any drug intoxication at any time. 1 week after treatment had been begun, most of the scabs about the base of the right ear, around both eyes, and over the face had scaled off, leaving a smooth, soft, elastic skin denuded of hair but otherwise quite healthy looking, and the involved area on the right hind foot had a similar appearance. The prepuce was practically normal and the testicles were rapidly becoming so.

The further changes in this rabbit during the succeeding 10 days were quite remarkable as are shown in Figs. 41 and 42. The eyelids, nose, face, and base of the right ear were no longer swollen or indurated and the rapid growth of hair in the bare areas was quite conspicuous. The affected area on the right hind foot was somewhat pinkish with a few yellowish pink scabs but with a beginning growth of fine hair. The external genitalia appeared normal, although on palpation the scrotum on both sides was still somewhat diffusely thickened and the testicles were atrophic. On the 11th and 14th days, two additional doses of 0.75 gm. per kilo of the drug were given intravenously for the purpose of determining any degree of acquired tolerance or any signs or symptoms of drug intoxication that these

additional large doses might cause, although from the point of view of the infection, we were confident that they were unnecessary. Apparently, however, they had no untoward effect whatever and the rabbit continued to improve very noticeably. By the end of the 3rd week, there were no residual lesions indicative of the trypanosomiasis infection except one or two small patchy areas below the inner canthus of each eye and just above the tip of the nose where the hair was thinner than elsewhere (Fig. 43), but in a short time, these areas had resumed a normal appearance. This particular rabbit has been held in the laboratory 3 years and there has been no return of the infection.

The treatment of trypanosomal relapses in rabbits with this compound depends largely upon two important factors, the general physical state of the rabbit and the extent and severity of the infection, and these conditions can in our opinion be most adequately handled by a system of repeated doses. Since the factor of tolerance for the amide of *N*-phenylglycine-*p*-arsonic acid is exhibited to a considerable degree in rabbits, the size of succeeding doses may be greatly increased if necessary and the number of dose repetitions may also be large. However, it is of essential importance that the succeeding doses should be repeated well before the effect of the previous dose has worn off.

Administration of the Drug by Routes Other than the Intravenous.

The great majority of experiments with the amide of *N*-phenylglycine-*p*-arsonic acid in the treatment of rabbit trypanosomiasis, including those described above, were carried out by the intravenous administration of the drug, since this route offers uniform and constant conditions from the point of view of actual administration as well as development of drug action. Under such conditions, a comparison of the effect of different sized doses and a final appraisal of the action of the drug from the point of view of a permanent cure may be more accurately made. However, since it may be desirable to employ routes of administration other than the intravenous, from the standpoint of actual usage and ease of manipulation, a few rabbits infected with *Tr. brucei*, of which the following are examples, were treated subcutaneously, intramuscularly, and *per os*, in order to ascertain the general effect and duration of certain selected doses upon initial infections and relapses.

Two rabbits with a 14 day nagana infection and showing well marked clinical signs of the disease were treated with 0.1 gm. of the drug per kilo of body weight subcutaneously. The acute inflammation and edema about the head and external genitalia were materially reduced and only slight signs remained 7 days after treatment, but on the 8th day, they became increased in the first rabbit. Consequently both animals were treated on the 8th and 10th days with 0.1 gm. per kilo, again subcutaneously. The clinical signs regressed completely in the first animal during the following week and were absent for 3 weeks, at which time they recurred with considerable severity; in the case of the second rabbit, in which the infection was still in abeyance at the time of the second and third doses, the clinical signs were absent for 6 days after the second treatment; they then recurred and steadily increased in severity and extent. Two other rabbits in this group were treated subcutaneously with a dose of 0.2 gm. of the drug per kilo of body weight which was not repeated. One rabbit was permanently cured and the other relapsed on the 36th day. A fifth animal with a long standing infection of 48 days duration showing marked chronic lesions about the head and external genitalia with considerable loss of weight was treated with 0.5 gm. of the amide of *N*-phenylglycine-*p*-arsonic acid per kilo subcutaneously. There were no signs of drug intoxication and no inflammation or induration about the site of injection; the regression of lesions proceeded somewhat more slowly than after intravenous administration of the drug, but at the end of 2 weeks they had subsided and the animal appeared normal except for a few bald patches about the head. No further signs of trypanosomiasis were observed for 41 days, at which time the outer margin of the right ear showed signs of early involvement and 2 days later a second dose of 0.25 gm. per kilo, one-half the original dose, was given subcutaneously for the purpose of ascertaining the effect upon the local lesion and upon the general course of the infection of a single dose much smaller than the original one. There was a very noticeable improvement of the ear lesion on the following day. A portion of the scab scaled off and the induration of the entire margin was distinctly decreased; on the 5th day after treatment, the ear appeared normal. There were no further indications of a relapse but unfortunately the rabbit developed an acute, extensive ringworm and

had to be killed 35 days after the second dose so that the question of a permanent cure is problematical. The rapid healing of the local ear lesion, however, and the fact that the relapse was treated soon after its appearance, although with a comparatively small dose, and that as long a time as 35 days elapsed without signs of a relapse either clinically or at autopsy are all factors indicative of a probable cure.

Seven rabbits were treated by the intramuscular route of administration. Two animals which were treated 14 days after inoculation with *Tr. brucei* received single doses of 0.2 gm. of the drug per kilo. The speed of action of the drug upon the well marked characteristic clinical lesions of the infection as seen in these two animals was apparently quite comparable with that observed after intravenous administration, and a permanent cure was effected in both rabbits. Two other rabbits of the same series were treated with an initial dose of 0.1 gm. per kilo which was repeated on the 8th and 10th days. One animal was cured but the other showed marked lesions of the disease 6 days after the last dose and was treated again with three doses of 0.1 gm. per kilo of body weight given intramuscularly on successive days with a marked, though temporary effect. In this instance, it is probable that the infection was originally not cleared by the first three doses and consequently either the second course of treatment was not given soon enough to effect a permanent cure or the amount of drug was not large enough. Another rabbit was treated 42 days after infection with *Tr. brucei* with three doses of 0.1 gm. per kilo of body weight intramuscularly on successive days. At the time of treatment, the lesions about the ears, eyes, face, nose, and lips were extremely marked and the animal itself was in only a fair physical condition. Regression and healing of the lesions about the head took place very satisfactorily and there were no signs of drug intoxication, but a subcutaneous abscess developed at the site of injection in the lumbar region and gradually increased in extent. The rabbit was killed 25 days after treatment. There were no clinical signs of a relapse at this time and the autopsy findings were negative. Finally, two rabbits with severe relapses after previous treatment—one with 0.015 gm. per kilo of neoarsphenamine given intravenously and the other with three doses of 0.1 gm. per kilo of the amide of *N*-phenylglycine-*p*-arsonic acid given subcutaneously—were treated intramuscularly with

larger doses of the latter drug. The first, which received 0.5 gm. per kilo of body weight, showed a rapid regression and healing of the lesions of the infection and was permanently cured; the other rabbit was given an initial dose of 0.3 gm. per kilo of body weight and 3 days later 0.5 gm. per kilo with no sign of drug intoxication and a very marked improvement in the signs of the infection. The ultimate result of the intramuscular administration of the drug cannot be determined in this case, for the succeeding treatment 11 days later was by mistake given intravenously.

Finally, an example of the therapeutic administration of the amide of *N*-phenylglycine-*p*-arsonic acid by mouth may be given for completeness, although the number of trypanosomiasis rabbits treated by this route is too small to admit of any conclusion regarding the effect and final result of various sized doses. A severe initial infection of 8 days duration with well marked clinical signs was treated with a single dose of 0.75 gm. per kilo of body weight given *per os* immediately after a small dose of sodium bicarbonate. There were no untoward effects following the administration of the drug; the clinical signs of the infection regressed and healed in the usual time and the rabbit was permanently cured.

While the number of rabbits treated with the amide of *N*-phenylglycine-*p*-arsonic acid administered by the subcutaneous, intramuscular, and *per os* routes is too few to admit of final conclusions as to their comparative value and usefulness, they demonstrate that even relatively small doses of the drug are highly active therapeutically when given intramuscularly and to a somewhat less extent subcutaneously, while in one instance a large dose given by mouth cured a severely infected animal.

SUMMARY.

In the treatment of experimental trypanosomiasis of rabbits with subsequent appraisal of the value of the therapeutic agent used, there are certain experimental factors including uniform infecting strains of trypanosomes and the observation of general procedures of method and time of inoculation conditioned by the infection itself which must be taken into account. The conspicuous and characteristic clinical

signs and symptoms seen in rabbit trypanosomiasis serve as criteria of the severity and duration of the disease, and it is obvious that the infection should be well established before treatment is instituted. For the same reason, before the question of a permanent cure can be established, treated rabbits should be kept under observation for a sufficient period of time, which with the species of organisms that we have used is at least 3 months.

The therapeutic results with the amide of *N*-phenylglycine-*p*-arsonic acid were obtained in rabbits which showed well marked clinical signs of a definitely established disease, and in many instances the infection was extremely advanced and of prolonged duration. The five species which we have employed, *Tr. brucei*, *Tr. gambiense*, *Tr. equinum*, *Tr. equiperdum*, and *Tr. evansi*, are uniformly fatal in rabbits. With the usual acute, actively progressing infection of from 1 to 2 weeks duration produced by our strain of *Tr. brucei*, the drug has a curative range of from 0.2 to 0.35 gm. per kilo of body weight, when administered intravenously in single doses, or from one-third to one-half the minimal lethal dose. Of the twenty-nine rabbits treated with doses falling within this range, twenty-five, or 86 per cent, were permanently cured and there were no relapses observed with doses above 0.3 gm. The infection produced by our strain of *Tr. gambiense* is controlled by a slightly lower dose, since there were no relapses with single doses of 0.3 gm. and a single dose of 0.15 gm. effected a cure in one of three rabbits so treated. The therapeutic experiments with *Tr. equinum*, *Tr. equiperdum*, and *Tr. evansi* are too few to admit of final conclusions, but apparently from the evidence at hand, much the same curative range is operative in *Tr. evansi* infections, while larger doses or a different system of treatment should have been employed in the treatment of rabbits infected with our strains of *Tr. equinum* and *Tr. equiperdum*.

In addition to the ultimate curative results obtained with single doses within the curative range, it is important to consider the marked therapeutic action with smaller single doses, as shown by the rapid regression and healing of the clinical lesions of the acute infections produced by all five species of trypanosomes together with a marked improvement in the general physical state of the animal. Moreover, large single doses, above those of the so called curative range, caused no disturbance of a toxic nature and were apparently well borne.

A system of repeated dose therapy may be employed with advantage in the treatment of both initial and relapsed infections in rabbits, especially in those instances in which there is induration or even necrosis of tissues with weakness and emaciation of the animal host. The factor of time of repetition or the spacing of doses is in our experience as important as that of size of the dose employed and depends upon the rate, degree, and duration of action of the particular dose of the drug in question. Since the amide of *N*-phenylglycine-*p*-arsonic acid apparently possesses the power of tissue penetration to a marked degree, it is desirable to give the second dose within a short time after the first in order that it may have a full opportunity for the immediate and complete development of its action. The repetition of small doses such as 0.15 gm. per kilo of body weight on successive or alternate days has given successful results as regards both the immediate regression and healing of lesions and ultimate permanent cures in severe, chronic infections. It is possible, however, to administer increasingly large doses, if this is necessary, since infected as well as normal rabbits exhibit a remarkable tolerance to repeated large doses of the drug. The therapeutic activity of small doses administered intramuscularly is quite comparable with that observed after similar doses given intravenously, as indicated by the rate of regression and healing of clinical lesions, while such effects proceed somewhat more slowly after subcutaneous injections. Permanent cures have been obtained in *Tr. brucei* infection with intramuscular and subcutaneous administration of single doses of from 0.2 to 0.5 gm. of the drug per kilo of body weight and in other instances with three repeated doses of 0.1 gm. per kilo given intramuscularly. One severely infected rabbit which received 0.75 gm. per kilo *per os* immediately following a small dose of sodium bicarbonate was also cured.

The therapeutic experiments here reported represent only a portion of those carried out with *N*-phenylglycineamide-*p*-arsonic acid and the scope of the present paper does not permit a detailed description of the many phases of the experiments or a full discussion of the various factors involved and the results obtained, all of which we hope to publish at some future time.

EXPLANATION OF PLATES.

The figures are reproductions of untouched photographs illustrating the effects produced upon experimental trypanosomiasis of rabbits by treatment with *N*-phenylglycineamide-*p*-arsonic acid. Objects are represented at about two-thirds their natural size, except where otherwise specified.

Figs. 1 to 17 illustrate the effect of single dose treatment upon acute and subacute initial infections.

Plate 17.

Fig. 1. *Tr. brucei.* Acute infection of 15 days duration. Day of treatment with 0.35 gm. per kilo of body weight given intravenously. There is marked swelling of the face, eyelids, and base of both ears. General condition of animal good.

Fig. 2. 1 week after treatment. Appearance of rabbit normal. Permanently cured.

Plate 18.

Fig. 3. *Tr. brucei.* Acute infection of 15 days duration. Objects represented at their natural size in Figs. 3 and 4. Day of treatment with 0.2 gm. per kilo of body weight given intravenously. The vulva and anus are markedly swollen and congested and are somewhat indurated. General condition of rabbit is good.

Fig. 4. 5 days after treatment. Rabbit permanently cured.

Fig. 5. *Tr. brucei.* Very severe, acute infection of 23 days duration. Day of treatment with 0.6 gm. per kilo of body weight given intravenously. The face, eyelids, and the lower portions of the ears are extremely swollen and indurated. The general physical condition of the rabbit is fairly good.

Plate 19.

Fig. 6. 1 week after treatment. Slight residual fullness of the face. The hair over the nose and both upper lids is coming off. Animal's general condition is good.

Fig. 7. 29 days after treatment. Permanently cured.

Plate 20.

Fig. 8. *Tr. gambiense.* Severe subacute infection of 36 days duration. Objects represented at their natural size in Figs. 8 to 11. Day of treatment with 0.2 gm. per kilo given intravenously. Both testicles are enlarged to about three times normal size and the scrotum is thickened, indurated, and cyanotic; on the right there are three large superficial ulcerations with scab formation. The testicles and cords are also enlarged and indurated. Rabbit's general condition is fairly good.

Fig. 9. 5 days after treatment. Scrotal scabs are separating.

Fig. 10. 19 days after treatment. There is a small residual adherent scab at the tip of the right scrotum. Testicle negative.

Fig. 11. 111 days after treatment—the day rabbit was discarded as permanently cured. External genitalia negative.

Plate 21.

Fig. 12. *Tr. equiperdum.* Severe subacute infection of 36 days duration. Day of treatment with 0.3 gm. per kilo of body weight given intravenously. Rabbit is very thin and weak. There is marked swelling of the face with moderate involvement of the ears. Both eyes, especially the right, are partially closed and the upper eyelids are swollen and reddened.

Plate 22.

Fig. 13. 5 days after treatment. There are no signs of trypanosomiasis and the animal's general condition is excellent. Ultimate recurrence.

Plate 23.

Fig. 14. *Tr. evansi.* Severe subacute infection of 36 days duration. Day of treatment with 0.3 gm. per kilo of body weight given intravenously. Both ears are involved nearly to the tip, the left more than the right; the face is swollen with thickening of the tissues over the bridge of the nose and of the lips. All four eyelids are swollen and reddened; there is a mucopurulent discharge from both eyes, which are almost closed, and numerous yellowish scabs adherent to the lids. General physical condition of rabbit is good.

Fig. 15. 5 days after treatment. Very marked improvement. General condition good.

Plate 24.

Fig. 16. 15 days after treatment. Scabs over end of nose and lips have desquamated. Beginning growth of hair.

Fig. 17. 19 days after treatment. The hair over the nose and lips is growing rapidly. Rabbit's condition is good with a considerable gain in weight. Permanently cured.

Plate 25.

Figs. 18 to 23 illustrate the effect of repeated dose therapy upon a chronic initial infection.

Fig. 18. *Tr. brucei.* Extremely severe chronic infection of 48 days duration. Day of beginning treatment with three doses of 0.2 gm. per kilo of body weight repeated at 24 hour intervals and given intravenously. There is a marked and extensive indurated swelling of the tissues of the ears, face, eyelids, nose, and

lips with a purulent exudate from the eyes and nose. Rabbit is weak and emaciated.

FIG. 19. 6 days after treatment had been begun. Marked improvement in the local clinical signs of the infection and in the rabbit's general condition.

PLATE 26.

FIG. 20. 6 days after treatment had been begun.

FIG. 21. 13 days after treatment had been begun.

PLATE 27.

FIG. 22. 21 days after treatment had been begun. There is a very marked growth of hair in the bald areas of the face and ears.

FIG. 23. 40 days after treatment. Permanently cured.

PLATE 28.

Figs. 24 and 25 illustrate the effect of single dose treatment upon an acute relapsed infection.

FIG. 24. *Tr. brucei.* Acute relapse of 7 days duration. Day of treatment with 0.5 gm. of the drug per kilo of body weight given intravenously. The entire left side of the face including the eyelids and ears is swollen. Good general physical condition.

FIG. 25. 1 week after treatment. Rabbit appears entirely normal. Permanently cured.

PLATE 29.

Figs. 26 to 44 illustrate the effect of repeated dose treatment upon acute, subacute, and chronic relapsed infections.

FIG. 26. *Tr. brucei.* Severe acute relapse of 8 days duration. Day of beginning treatment consisting of three doses of 0.15 gm. per kilo of body weight repeated at 24 hour intervals and given intravenously. The face is markedly swollen and indurated and both eyes are closed. The base of both ears is similarly involved. General condition of the rabbit is fair.

FIG. 27. 6 days after treatment had been begun. Slight residual swelling over the bridge of the nose. General condition excellent. Probable permanent cure.

PLATE 30.

FIG. 28. *Tr. brucei.* Severe relapse of 15 days duration on the day of beginning treatment with 0.5 gm. per kilo of body weight given intravenously and repeated 14 days later. There is marked involvement of the face, ears, eyelids, nose, and lips and a mucoserous nasal discharge. Animal very thin and weak.

FIG. 29. 49 days after treatment. Appearance of rabbit normal except for the twisting of the head to the right. Probable permanent cure.

PLATE 31.

FIG. 30. *Tr. brucei.* Severe relapse of 22 days duration on the day of beginning treatment with 0.4 gm. per kilo of body weight given intravenously. The face, base of both ears, eyelids, nose, and upper lips are markedly swollen and edematous and there are thick scabs on the lips.

FIG. 31. 7 days after treatment had been begun. There is a marked improvement in the clinical signs.

PLATE 32.

FIG. 32. 9 days after treatment had been begun. Improvement continues with increase of weight.

FIG. 33. 15 days after treatment had been begun. Clinical signs of the infection have recurred.

PLATE 33.

FIG. 34. 21 days after treatment had been begun. Marked improvement with desquamation of the scabs and necrotic tissue in the involved area. General condition excellent.

FIG. 35. 28 days after treatment had been begun. Further improvement with rapid return to normal appearance. Ultimate relapse.

PLATE 34.

FIG. 36. *Tr. brucei.* Advanced chronic relapse of about 3 months duration on the day of beginning treatment with repeated doses of 0.75 gm. per kilo of body weight given intravenously. There is marked involvement of the face, eyelids, base of ears, and nose with loss of hair and scab formation. Rabbit is weak but not markedly emaciated.

FIG. 37. *Tr. brucei.* Day of treatment. Objects represented at their natural size. The prepuce is markedly swollen and indurated; the testicles and scrotum are very firm and indurated although not enlarged.

PLATE 35.

FIG. 38. 3 days later.

FIGS. 39 and 40. 1 week later. The appearance of the head and the external genitalia is normal except for the absence of hair and a few tiny scabs around the right eye. In Fig. 40 objects are represented at their natural size.

PLATE 36.

FIG. 41. 12 days later. There is a growth of fine hair on the bald patches about the head.

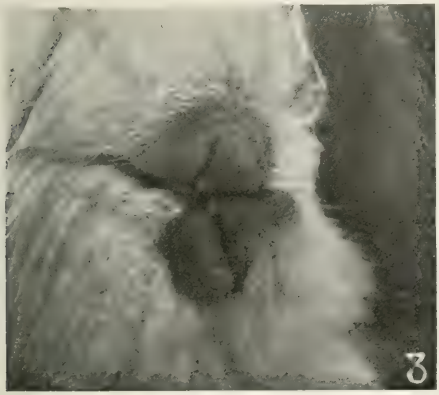
FIG. 42. 17 days later. Rapid growth of hair continues.

PLATE 37.

FIG. 43. 22 days later. Rabbit's appearance is entirely normal except for a tiny bald spot below the inner canthus of the right eye.

FIG. 44. 1 year later. Animal permanently cured.

(Pearce and Brown: Trypanosome and spirochete infections.)



(Pearce and Brown: Trypanosome and spirochete infections.)

(Pearce and Brown: Trypanosome and spirochete infections.)

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(Pearce and Brown: Trypanosome and spirochete infections.)

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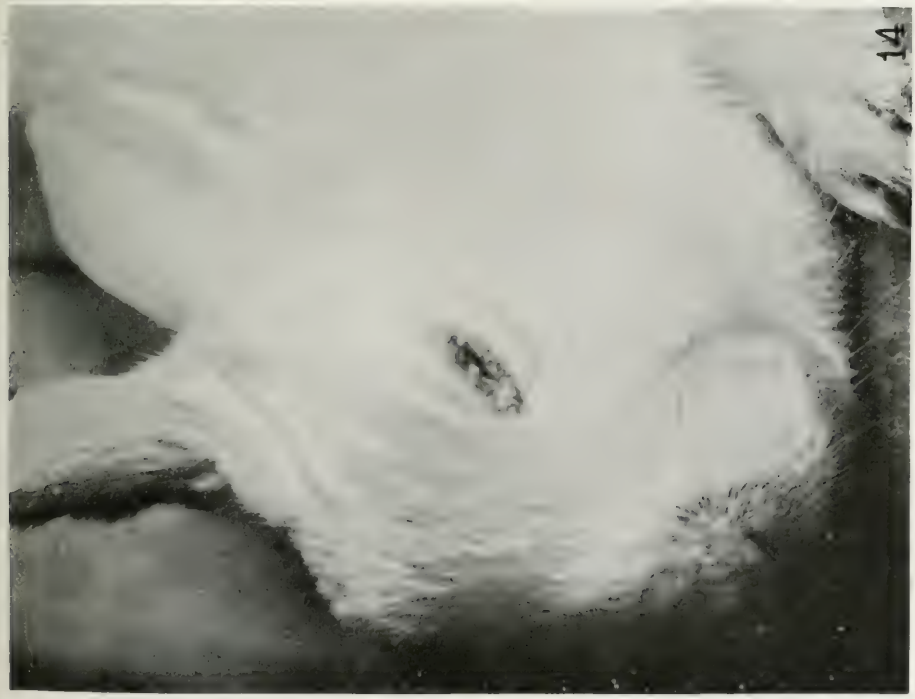
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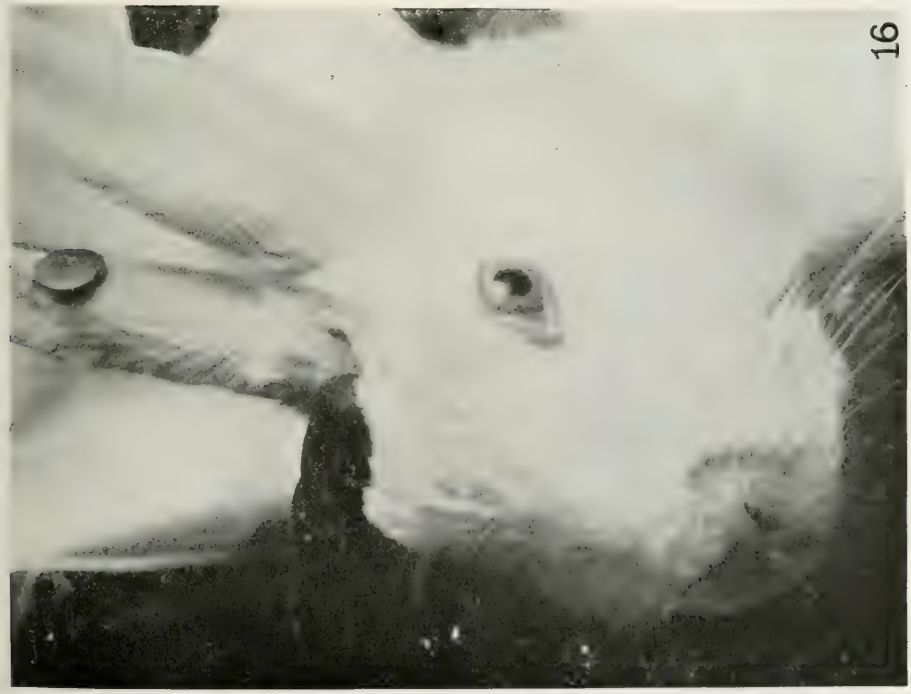
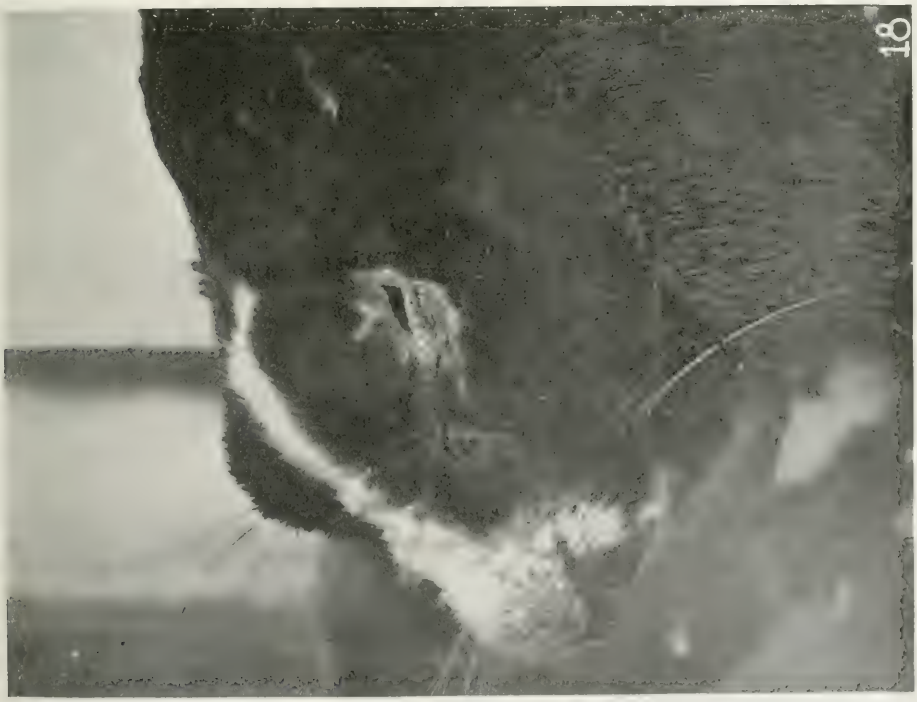


(Pearce and Brown: Trypanosome and spirochete infections.)



(Pearce and Brown: Trypanosome and spirochete infections.)

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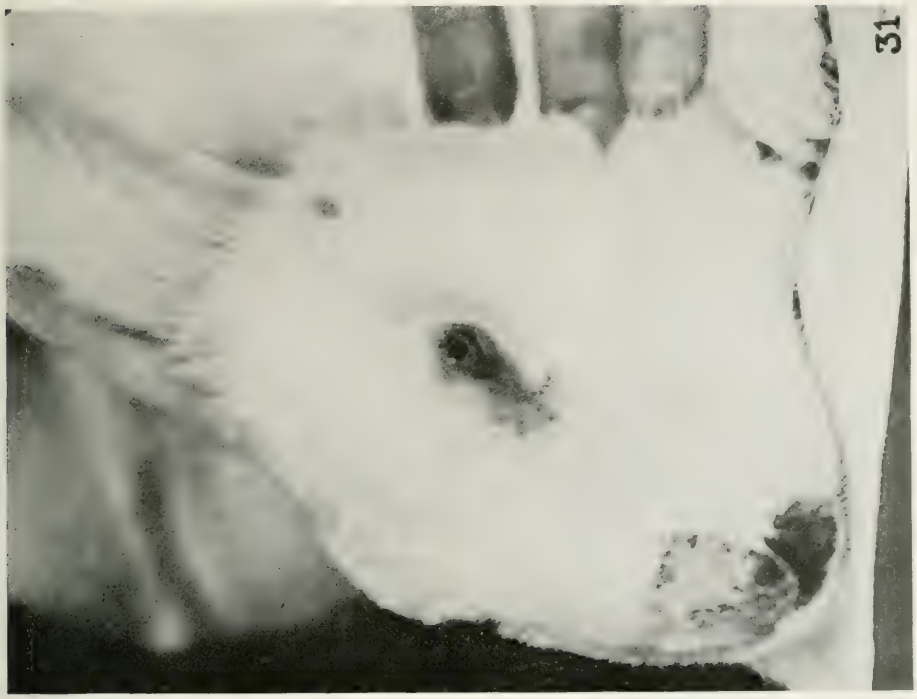
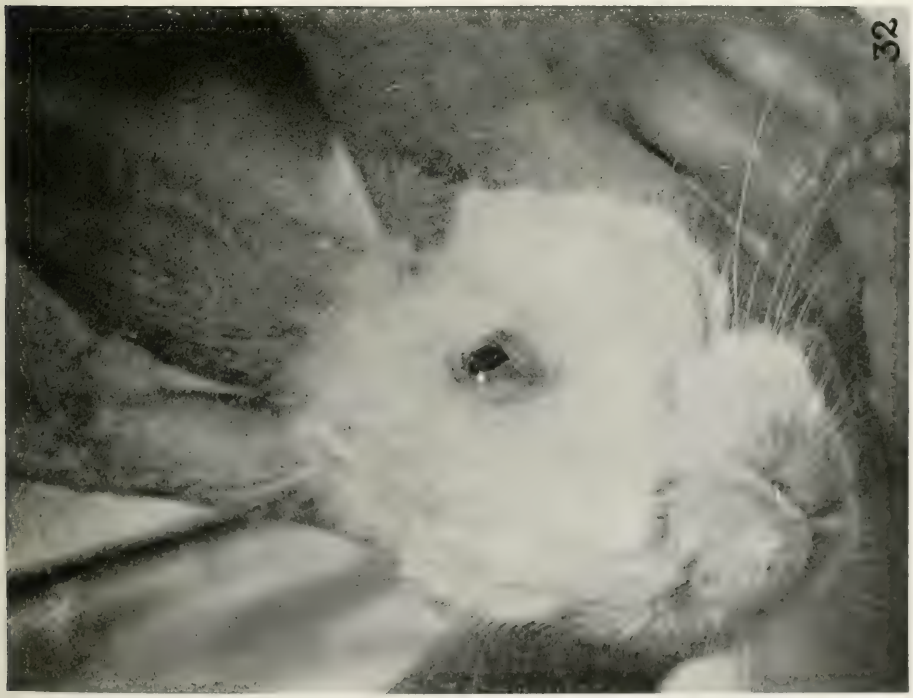
(Pearce and Brown: Trypanosome and spirochete infections.)

(Pearce and Brown: Trypanosome and spirochete infections.)

(Pearce and Brown: Trypanosome and spirochete infections.)



(Pearce and Brown: Trypanosome and spirochete infections.)



(Pearce and Brown: Trypanosome and spirochete infections.)

(Pearce and Brown: Trypanosome and spirochete infections.)

(Pearce and Brown: Trypanosome and spirochete infections.)

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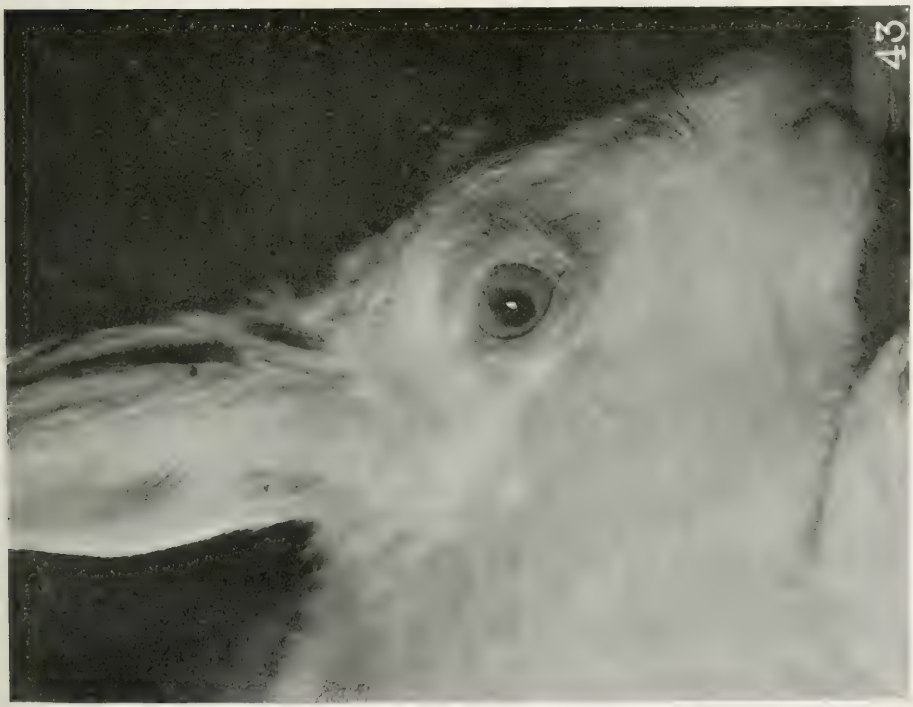
(Pearce and Brown: Trypanosome and spirochete infections.)



(Pearce and Brown: Trypanosome and spirochete infections.)

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CHEMOTHERAPY OF TRYPANOSOME AND SPIROCHETE INFECTIONS.

Biological Series. IV.

The Action of N-Phenylglycineamide-*p*-Arsonic Acid upon Spirochete Infections.

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Plates 38 to 44.

(Received for publication, June 18, 1919.)

A second group of conditions upon which the action of *N*-phenylglycineamide-*p*-arsonic acid has been studied is the infections produced in laboratory animals by spirochetes of the *recurrens* group and by *Treponema pallidum*. Early in the course of these studies, it was found that the action of the drug upon these organisms was not so pronounced as in the case of the trypanosomes. Accordingly, the experiments which were carried out were designed more for the purpose of studying the range and character of the action than as attempts to cure the infections.

Blood Spirochetes.

The action of A 63 upon the spirochetes of relapsing fever was studied in both rats and mice infected with *Sp. obermeieri* and with *Sp. novyi*. The animals for these experiments were inoculated intraperitoneally with blood suspensions of the organisms given in such doses as would produce an infection of the desired character—usually an infection which would show a few spirochetes in the peripheral blood within 24 hours after inoculation. The animals were then treated by intraperitoneal injection of single doses of the drug and the results followed from day to day by examination of tail blood under dark-field illumination. Finally, at the end of 60 days, surviving

animals were reinoculated as a means of determining the presence or absence of immunity such as usually develops in the course of untreated infections which terminate in spontaneous recovery, or in treated animals which have not been cured, and is absent in those which have been cured.

The results obtained from the treatment of rats infected with either of the spirochetes mentioned were almost entirely negative. The blood of these animals could not be cleared of spirochetes except by the use of doses of the drug which were nearly always fatal (1.5 gm. per kilo), while smaller doses exercised very little influence upon the course of the infection.

The action of A 63 appeared to better advantage in mice, however, due perhaps to the greater tolerance of these animals for the drug. With an average infection, the dose required to clear the blood of spirochetes temporarily was 1.5 gm. per kilo, or approximately the same as that found to be necessary in the case of rats, but no lasting effect was produced until the dose of the drug used reached upwards of 2 gm. per kilo of mouse, or the largest dose which could be used with safety.

The general character of the therapeutic effects produced may be seen by reference to Table I which shows the results obtained from the treatment of one series of mice with doses of A 63 ranging from 1.5 to 2.25 gm. per kilo. The results of blood examinations are given for the 1st week only with the final result obtained from reinoculation at the end of 60 days.

The infection in these mice might seem to have been a rather severe one since all four controls died within 8 days but the results obtained with the treated mice were of an average character. It will be seen from an examination of this table that the course of the infection was modified in all cases. Usually the progress of the infection was checked rather abruptly and the peripheral blood freed of spirochetes within 24 hours, or by crisis as it were. In a few instances (Mice 6 and 12), this effect was accomplished more slowly; the progress of the infection was first arrested and the infection then terminated by lysis instead of by crisis. In still other animals, the effect of the drug was manifested only by a reduction in the severity of the infection as indicated by the numbers of organisms in the peripheral blood. These effects were not always lasting but were followed in some instances by one or more slight relapses.

When the surviving mice were reinoculated at the end of 60 days, an interesting condition was found in that some mice known to have shown a recurrence of spirochetes in the peripheral blood were as susceptible to reinoculation as those in which no relapse had been observed. While the untreated controls in this experiment were all

TABLE I.

Results Obtained from the Treatment of a 24 Hour Infection of Sp. obermeieri in Mice.

Dose per kilo.	No. of mouse.	Results of blood examinations on days following inoculation and treatment.								Blood on days after reinoculation on 60th day.	
		1	2	3	4	5	7	8	60	2	3
gm.											
2.25	1	+	−	−	−	−	−	−	−	+	++
	2	+	−	−	−	−	−	++	−	+	++
	3	+	−	−	−	−	−	−	D.		
	4	+	−	−	−	−	+	−	"		
	5	+	−	−	−	−	D.				
2.0	6	+	+	+	−	−	−	−	−	−	++
	7	+	−	−	−	−	−	−	−	−	++
	8	+	−	−	−	−	−	−	−	−	++
	9	+	−	−	−	−	−	−	−	−	+
	10	+	−	−	−	−	+	+	−	−	++
1.75	11	+	−	−	−	−	D.				
	12	+	+	+	−	−	+	−	D.		
	13	+	−	−	−	−	++	−	"		
	14	+	−	+	++	−	−	−	"		
	15	+	−	−	−	−	−	−	"		
1.5	16	+	−	−	−	−	++	−	−	−	−
	17	+	+	++	−	−	−	−	−	−	−
	18	+	−	++	−	−	++	++	−	−	−
Controls.	19	+	+++	++++	++++	−	D.				
	20	+	+++	++++	++++	−	"				
	21	+	+++	++++	++++	−	−	D.			
	22	+	+++	++++	++++	−	−	"			

In the tables − indicates no spirochetes found, + few spirochetes present, ++ spirochetes fairly numerous, +++ spirochetes numerous, ++++ spirochetes present in large numbers, D. dead.

dead, the negative results obtained with the mice given only 1.5 gm. of the drug per kilo served as checks against the positive results obtained with the other animals.

Relapse and spontaneous recovery without immunity are almost a paradox, but there are a number of suggestions which occur to one as to how or why such anomalous conditions might arise. Whatever the explanation offered, however, it is obvious that the drug has had its effect even in these relapses and that any immunity developed was either so slight or of such short duration that these mice reacted to reinoculation after 2 months, just as if they had been promptly and completely freed of the spirochetes by the administration of the drug.

In estimating the curative effects of A 63, therefore, one can speak with assurance only of the results of reinoculation. On this basis, it was found that mice infected with *Sp. obermeieri* could be cured with doses of A 63 ranging from 1.5 gm. per kilo of mouse upwards, but with no dose of the drug were we able to obtain cures in more than 75 per cent of the animals treated.

Treponema pallidum.

The action of drugs upon syphilitic infections has usually been studied by the use of some form of the infection which can be produced in rabbits by inoculation with *Treponema pallidum*, and these experimental infections have been used in various ways by different investigators. The lesions of the eye, testicle, and skin have all proved of some value but the scrotal chancre is generally regarded as the most serviceable lesion of the group. The effects produced by drugs upon these infections may be estimated conveniently in three ways: first, by the changes produced in the lesions themselves; second, by the effects upon the organisms present in the lesions; and finally, by the duration or permanence of any changes which may be induced.

In this way, the action of A 63 upon scrotal chancres was studied in a series of twenty-nine rabbits. The lesions were measured with calipers and in most instances were photographed before treatment and at various intervals thereafter in order to determine as accurately as possible the character, extent, and rate of the changes which took place. In like manner, the lesions were aspirated and the fluid was

examined by dark-field illumination before treatment and at intervals such as 1, 3, 5, 7 days, etc., after treatment as a means of following the effects upon the spirochetes. The treated animals were kept under constant observation for such periods of time as the purpose of the experiment demanded, which varied from 1 to 7 months with different animals. For the most part, treatment was carried out by the use of single doses of the drug given intravenously; one series of animals was treated by subcutaneous administration, a second by intramuscular, and a third by the use of repeated small doses of the drug given intravenously. Some study was also made of the action of the drug when given by mouth.

The nature of the results obtained from these experiments may be seen by reference to Table II which contains the abbreviated protocols of twelve rabbits treated by intravenous administration of single doses of A 63 ranging from 0.1 to 0.5 gm. per kilo of body weight.

In order to make these results more intelligible, certain details of the experiments must be supplied. Rabbit 1 of this series was inoculated November 20, 1916. The chancres developed rapidly and at the end of 50 days when treatment was carried out, they measured 2.4 by 2.4 by 1.5 cm. (8.64 cc.) and 2.6 by 2.3 by 1.6 cm. (9.568 cc.) on the right and left respectively. On the day following treatment, the chancres were somewhat edematous, and while a few non-motile spirochetes could still be found, they had disappeared entirely by the end of 48 hours and were never found again. The chancres regressed rapidly for 2 weeks after treatment, when the crusts were torn from the ulcers by the animal. The wounds became secondarily infected and healing was somewhat delayed. The animal was still negative when discarded 100 days after treatment.

The second rabbit of this group requires but little comment. It was an animal with moderately large chancres which reacted to treatment precisely as did Rabbit 1. This animal died from hemorrhagic septicemia 6 days after treatment.

The next two rabbits of the series present an important contrast in the effects produced by the drug. Rabbit 3 was inoculated November 20, 1916, and treated January 9, 1917. At the time of treatment the chancres measured 1.7 by 1.55 by 1.25 cm. (3.294 cc.) on the right and 2.1 by 1.8 by 1.5 cm. (5.67 cc.) on the left. They were markedly

TABLE II.

Results Obtained from the Treatment of Rabbits Infected with T. pallidum.

Dose per kilo.	No. of rabbit.	Testicle.	Chancres at time of treatment.		Results of treatment.						Length of observation.
					Spirochetes.				Chancres.		
			Volume.	No. of spirochetes.	24 hrs.	1 wk.	2 wks.	Recurrence.	Time of healing.	Recurrence.	
gm.			*cc.*						*wks.*		*days*
0.5	1	Right.	8.64	++	−	−	−	No.	5	No.	100
		Left.	9.568	++++	+	−	−				
	2	Right.	5.304	+++	−	D.					
		Left.	6.16	++	−						
0.4	3	Right.	3.294	+++	−	−	−	No.	3	No.	207
		Left.	5.67	++++	−	−	−				
	4	Right.	8.1	++++	−	−	−	Yes.	5	No.	147
		Left.	6.799	+++	+	−	−				
0.3	5	Right.	1.89	++++	−	−	−	No.	3	No.	207
		Left.	3.315	+++	−	−	−				
	6	Right.	3.956	+++	−	−	−	Yes.	3	No.	207
		Left.	2.925	++++	−	−	−				
0.2	7	Right.	1.3	+++	−	−	−	No.	3	No.	85
		Left.	3.22	++++	+	+	−				
	8	Right.	3.47	++++	+	+	−	Yes.	Not healed.	No.	85
		Left.	3.31	++++	+	−	−				
	9	Right.	4.817	++++	+	−	+++		D.		
		Left.	2.755	++++	+	++	+				
	10	Right.		++			+	Never clear.	3	On left.	D.
		Left.	3.27	+++	+++	+	++				
0.1	11	Right.	2.38	+++	+++	−	−	Yes.	2	Yes.	72
		Left.		No lesion.							
	12	Right.	1.586	+++	++	+	++	Never clear.	Not healed.	Yes.	
		Left.	1.42	+++	++		+				

indurated and growing very actively (Fig. 1). Spirochetes were numerous. On aspiration 24 hours later, there was a marked increase in the fluid content of the lesions and no spirochetes could be found nor were they observed at any subsequent examination. The lesions regressed rapidly and at the end of 3 weeks were completely healed with moderate residual thickening in the region of the scars on both sides (Figs. 1 to 4). This rabbit was kept under constant observation for 207 days during which time no lesions, local or general, were observed.

Rabbit 4 was treated 40 days after inoculation. On the day of treatment, the chancres measured 2.5 by 1.8 by 1.8 cm. (8.1 cc.) on the right and 2.1 by 1.85 by 1.75 cm. (6.799 cc.) on the left and were of essentially the same character as those of Rabbit 3 (Fig. 5). For the first 2 weeks, the effects of treatment appeared to be about the same as in Rabbit 3. Regression then proceeded more slowly and the lesions were not completely healed until the end of the 5th week (Figs. 5 to 8), and while there was considerable residual thickening about the scars, no spirochetes could be found. This diffuse thickening gradually diminished, but translucent, glistening, and slightly indurated patches developed at the site of the scars (Fig. 9), and on the 52nd day, numerous actively motile spirochetes were found in these patches. This condition persisted with but slight change for several weeks and then cleared up, as shown in Figs. 9 and 10. The point to be noted here is that although spirochetes were present in the scrotum of this animal at the site of the original lesions, the associated lesions were very slight and showed almost no tendency to growth.

Coming to a still smaller dose of the drug, we again have two animals in which the effects produced were somewhat different. Rabbit 5 was treated 50 days after inoculation. The chancres at this time measured 1.4 by 1.35 by 1 cm. (1.89 cc.) and 1.7 by 1.5 by 1.3 cm. (3.315 cc.) on the right and left respectively. They were both actively growing and well indurated chancres; the one on the right showed a small depressed ulcer, while that on the left was not ulcerated. Spirochetes were numerous on both sides. The results of treatment were essentially the same in all respects as those with Rabbit 3. During 207 days observation, neither spirochetes nor lesions of any kind could be found.

Rabbit 6 was inoculated on November 24, 1916, and treated 46

days later. The chancres in this animal measured 2.15 by 1.6 by 1.15 cm. (3.956 cc.) and 1.95 by 1.5 by 1 cm. (2.925 cc.). Spirochetes disappeared and healing of the chancres took place in the usual manner. There was no sign of relapse for 7 weeks after treatment when several small nodules not more than 1 mm. in diameter appeared in the region of the scar and along the vessels in the dorsal fold of the scrotum on the left. Puncture of these nodules showed numerous actively motile spirochetes. Similar lesions developed later on the right. This animal was kept under close observation for 23 weeks after these lesions appeared, during which time the lesions showed very little change, until they finally regressed and disappeared.

In the next group of rabbits, there were four animals which gave a variety of results. Rabbit 7 responded to treatment by a gradual disappearance of the spirochetes and by rapid regression and healing of the lesions which was almost complete in 3 weeks (Figs. 11 to 14), and remained negative during a period of 85 days observation.

Rabbit 8 also showed a reduction in the spirochetes, and for a short time near the end of the 2nd week after treatment, no spirochetes could be found. In the meantime the chancres had decreased to less than half their original size, but at this point regression ceased. The chancres did not grow again, however, but after remaining stationary for a week or so, underwent spontaneous regression.

Rabbits 9 and 10 behaved still differently. At no time were the lesions of these animals free from spirochetes with the exception of one chancre of No. 9. Nevertheless, the chancres all regressed at a normal rate, and in No. 10, they were healed at the end of 3 weeks with slightly thickened, glistening patches at the site of the scars (Figs. 15 to 18). This rabbit lived only 36 days after treatment, but during this time, there was no renewal of the growth of the lesions, although numerous actively motile spirochetes were present all the while. The other rabbit, No. 9, died of hemorrhagic septicemia before the chancres had healed.

The final group of two rabbits gave results of much the same character as those of the preceding group. Rabbit 11 was inoculated November 1, 1916, but there was very little reaction for 2 months. The chancre on the right then developed rapidly and when treatment was carried out 97 days after inoculation (or about 40 days after the

beginning of the specific reaction) the main chancre mass measured 1.85 by 1.14 by 1.13 cm. (2.38 cc.). On the left, there was only a tiny nodule. Spirochetes disappeared from the chancre in 1 week after treatment and the lesion was healed with moderate residual thickening at the end of 2 weeks (Figs. 19 to 21). The animal was observed for 72 days during which time a small nodule developed in the deeper tissues of the scrotum beneath the scar. A similar nodule also appeared in the left scrotum but neither of these nodules grew and had almost disappeared at the time the animal was discarded.

Rabbit 12 was inoculated December 18, 1916, and treated 49 days later. The chancre on the right measured 1.48 by 1.24 by 0.87 cm. (1.586 cc.) and that on the left 1.41 by 1.2 by 0.84 cm. (1.42 cc.). They were both well indurated and actively growing chancres (Fig. 22). The effect of treatment was to reduce the spirochetes to a moderate extent and to cause considerable regression of the lesions lasting over a period of about 12 days (Figs. 23 and 24). Both chancres then began to increase in size (Fig. 25), in which respect the result differed from that usually obtained from the use of larger doses of the drug.

To the effects which have been described, one other type of reaction should be added; namely, that of the refractory animal or the refractory infection, which was encountered twice in our series of twenty-nine rabbits. The best example which we have of the infection which did not yield to treatment is that shown in Figs. 26 to 29. This animal was inoculated November 8, 1916. For 2 months, the growth of the chancres was rather slow and irregular, but after that they developed very rapidly and measured 1.82 by 1.57 by 1.3 cm. (3.715 cc.) and 1.75 by 1.7 by 1.35 cm. (4.016 cc.) 87 days after inoculation. Both chancres were markedly indurated and contained unusually large numbers of spirochetes. The rabbit was given three intravenous injections of A 63, 48 hours apart, each dose representing 0.1 gm. per kilo. The effect of this treatment was comparatively slight. The spirochetes were very little affected, but the chancres diminished in size for about 9 days with a maximum decrease of 0.4 cm. in all dimensions. They then began to increase in size and 3 weeks after treatment (Fig. 28) measured 1.67 by 1.93 by 0.9 cm. (2.901 cc.) and 1.5 by 1.37 by 0.9 cm. (1.85 cc.). The rabbit was then treated for the second time with a single dose of 0.3 gm. given intramuscularly

with an effect which was but slightly greater than that of the first treatment, the extent of which is shown in Fig. 29.

While the effects which have been described relate almost entirely to intravenous therapy, much the same results were obtained when the drug was given either subcutaneously or intramuscularly. The chief difference noted was that slightly larger doses of the drug were required by either of these routes to produce effects comparable with those obtained by intravenous administration. The drug was found to be active also when given by mouth, but this method of treatment was not used to a sufficient extent to warrant any statement as to its relative value.

Finally, one series of rabbits was treated by the use of repeated small doses of A 63 given intravenously, mainly for the purpose of getting an idea of the size of the dose and the interval between doses which would be required to maintain a continuous therapeutic effect. Without going into the details of these experiments, it may be said that the smallest dose which appeared to exercise a definite influence upon the infection was 0.1 gm. per kilo and that such doses could not be spaced more than 48 hours apart if the effect was to be made continuous. The experiments were not carried far enough to determine just how many such doses would be required to produce a given end-result.

From these experiments one may gain a fair impression of the effects produced by the amide of *N*-phenylglycine-*p*-arsonic acid upon experimental infections of *Treponema pallidum*. As seen by examination of fluid drawn from the lesions, the effect upon the spirochetes is to produce an impairment of motility or a complete loss of motion followed by degeneration and gradual disintegration so that the spirochetes eventually disappear from the lesions if the dose used is sufficiently large. One may be reasonably certain, therefore, that the drug possesses some measure of spirocheticidal action, but the dose required to produce such an effect as that described is comparatively large. At least 0.1 gm. per kilo is necessary to produce any appreciable effect upon the spirochetes; and the effect is usually not very pronounced until the dose used reaches upwards of 0.3 gm. per kilo, and even 0.4 gm. does not always insure a permanent disappearance of the infecting organisms.

The effect of the drug upon the lesions themselves is much more definite and apparently out of proportion to the effect produced upon the spirochetes. As we have seen, complete resolution and healing of scrotal chancres may be accomplished with doses as small as 0.1 gm. per kilo, and this is the usual result obtained with doses as large as 0.2 to 0.3 gm. whether spirochetes are destroyed or not. Further than this, the effects, as far as the lesions are concerned, appear to be more enduring. In our experience, lesions which have once healed, have shown little or no tendency to recur even though actively motile spirochetes were present. In a way, this peculiar type of action reminds one of the process of spontaneous control of *pallida* infections in rabbits; but it is not the same, and whatever the explanation of these effects may be, they are definitely referable to the action of the drug.

CONCLUSIONS.

To summarize the results obtained from these experiments, one may say that *N*-phenylglycineamide-*p*-arsonic acid is capable of exercising a very definite effect upon the course of infections produced by spirochetes of the *recurrens* group and by *Treponema pallidum*. It is more difficult to say, however, just how these effects should be interpreted. In the case of the blood spirochetes, the infection is ameliorated; and even though the spirochetes are not immediately destroyed, the infection is frequently brought to a termination which leaves the animal in a condition not unlike that produced by more powerful spirocheticidal agents. That is, the infecting organisms are either affected in such a way that they eventually die off or are destroyed by the host in such a way that no lasting immunity is developed in consequence of their destruction.

Apparently much the same type of reaction occurs in the treatment of rabbits infected with *Treponema pallidum*. It is possible that when very large doses of the drug are used, these organisms may be completely destroyed, but it is certain that in other cases, where complete healing of the lesions is accomplished as a result of treatment, the organisms are not destroyed. Moreover, it appears that such a result can be accomplished in the presence of numerous actively motile spirochetes, and once the effect of the drug has reached this

point, either the capacity of the spirochetes for stimulating reaction on the part of the tissues is lowered or else the reactivity of the tissues is reduced. At any rate, living spirochetes may remain in the tissues for considerable periods of time without giving rise to the usual tissue reaction which characterizes these infections.

With either group of organisms, therefore, *N*-phenylglycineamide-*p*-arsonic acid appears to act in a manner somewhat different from that of the usual spirocheticidal agents. While it does possess a considerable degree of spirocheticidal action, its chief effect is seen in the peculiar manner in which it modifies or controls the course of these infections.

Further than this, we shall not attempt to go at present. As set forth in the literature, the facts and principles upon which such experiments depend are so few as to offer almost no basis for an interpretation of these experiments. When we have had an opportunity of reporting our own experience in dealing with these infections, we may return to a consideration of the facts here reported.

EXPLANATION OF PLATES.

The figures are reproductions of untouched photographs illustrating the effects produced upon scrotal chancres of rabbits by treatment with *N*-phenylglycineamide-*p*-arsonic acid. Objects are represented at their natural size.

PLATE 38.

FIGS. 1 to 4. Rabbit 3. Effect produced by 0.4 gm. per kilo of body weight.

FIG. 1. Chancres at time of treatment.

FIG. 2. 1 week after treatment.

FIG. 3. 2 weeks after treatment.

FIG. 4. 3 weeks after treatment. The lesions are healed with some residual thickening especially noticeable on the right, but no spirochetes could be found.

PLATE 39.

FIGS. 5 to 10. Rabbit 4. A peculiar therapeutic effect following the administration of a dose of 0.4 gm. per kilo.

FIG. 5. Chancres at the time of treatment.

FIG. 6. Chancres regressing rapidly.

FIG. 7. 4 weeks after treatment. Chancres unhealed but still regressing slowly.

FIG. 8. 5 weeks after treatment. Chancres are healed but the scars are thickened and translucent, especially that on the right.

FIG. 9. 9 weeks after treatment. The lesions are still healed but scars are now represented by translucent, highly refractile plaques which contain numerous actively motile spirochetes.

FIG. 10. 147 days after treatment. The foci of infection have cleared spontaneously and the scrotum is normal.

PLATE 40.

FIGS. 11 to 14. Rabbit 7. The effect of a single dose of 0.2 gm. per kilo.

FIG. 11. Chancres at the time of treatment. That on the right is an unulcerated globular mass.

FIG. 12. 1 week after treatment; very rapid regression of the lesions.

FIG. 13. 3 weeks after treatment. The chancre on the right has entirely disappeared; that on the left still shows a small thickened mass.

FIG. 14. 4 weeks after treatment. Resolution complete.

PLATE 41.

FIGS. 15 to 18. Rabbit 10. An instance of the healing of chancres with a single dose of 0.2 gm. per kilo while actively motile spirochetes were constantly present.

FIG. 15. Chancres at the time of treatment.

FIG. 16. 1 week after treatment; marked regression of the lesions.

FIG. 17. 2 weeks after treatment.

FIG. 18. 3 weeks after treatment. The lesions have practically disappeared, but actively motile spirochetes are fairly numerous in the scars of both sides. There was a slight recurrent patch in the left scrotum.

PLATE 42.

FIGS. 19 to 21. Rabbit 11. An effect which may be accomplished by a dose of 0.1 gm. in lesions which are easily influenced.

FIG. 19. Chancre at the time of treatment.

FIG. 20. 1 week after treatment.

FIG. 21. 2 weeks after treatment.

PLATE 43.

FIGS. 22 to 25. Rabbit 12. The extent of the effect usually produced by a dose of 0.1 gm. per kilo.

FIG. 22. Chancres at the time of treatment.

FIG. 23. 8 days after treatment.

FIG. 24. 2 weeks after treatment. The chancre on the left is healed, but there is a considerable mass of unresolved tissue in the scrotum; that on the right is increasing.

FIG. 25. 3 weeks later. Both chancres growing actively.

PLATE 44.

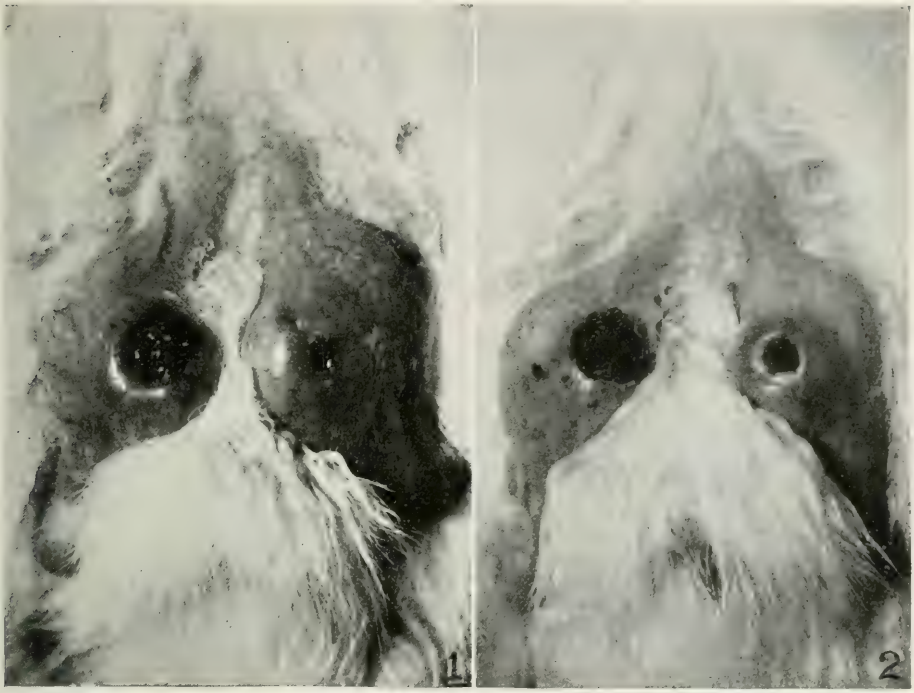
FIGS. 26 to 29. Chancres which proved refractory to treatment with this drug.

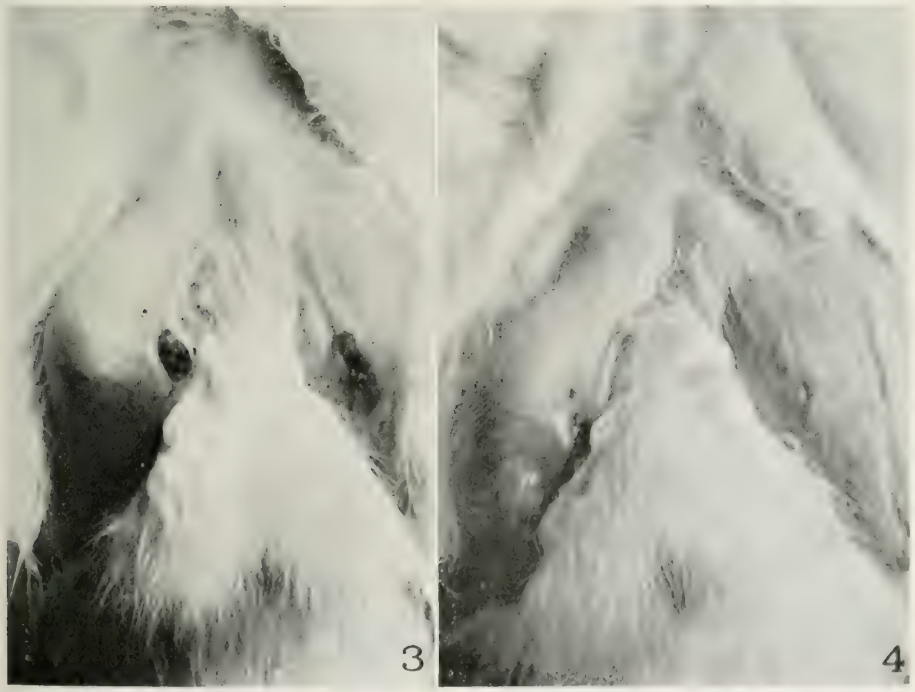
FIG. 26. Chancres at the time of first treatment which consisted of three doses of 0.1 gm. per kilo given intravenously at intervals of 48 hours.

FIG. 27. 1 week after treatment had been commenced; practically the full extent of the reduction of the lesions is shown.

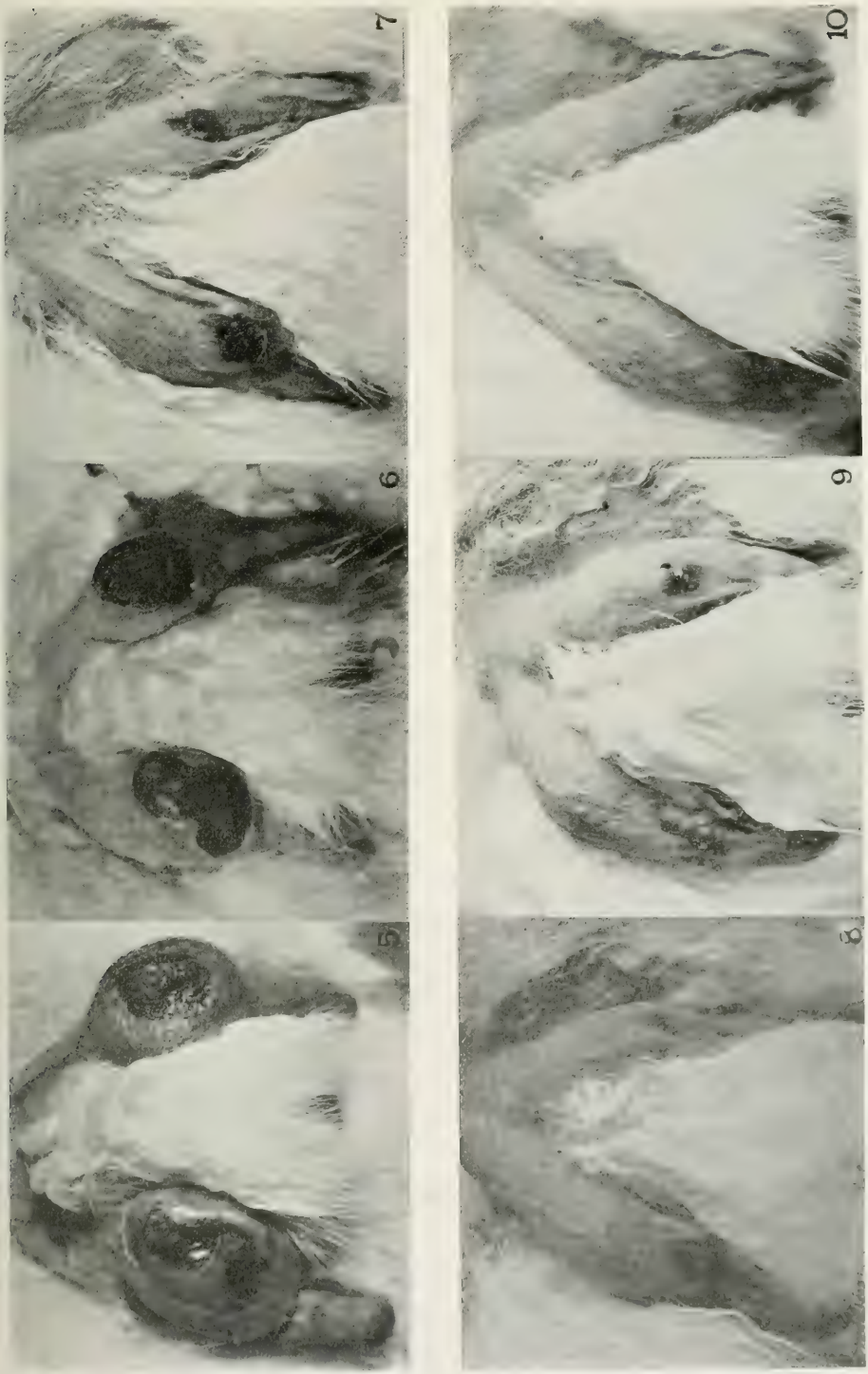
FIG. 28. 2 weeks later; the growth of the chancres in the interim is shown. Animal retreated with 0.3 gm. per kilo given intramuscularly.

FIG. 29. 1 week later; a decided effect is shown, but renewed activity set in almost immediately.





(Brown and Pearce: Trypanosome and spirochete infections.)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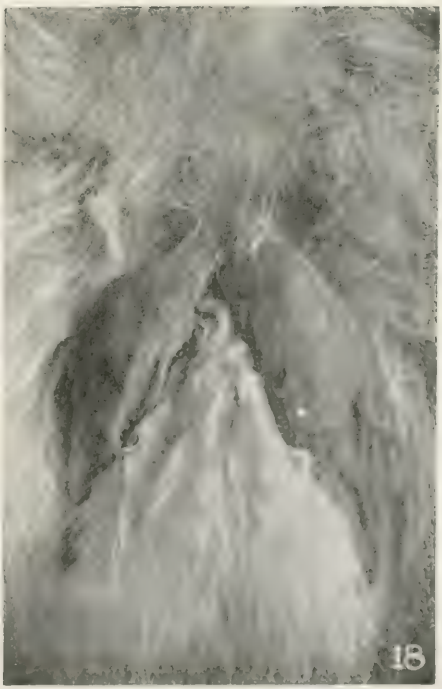
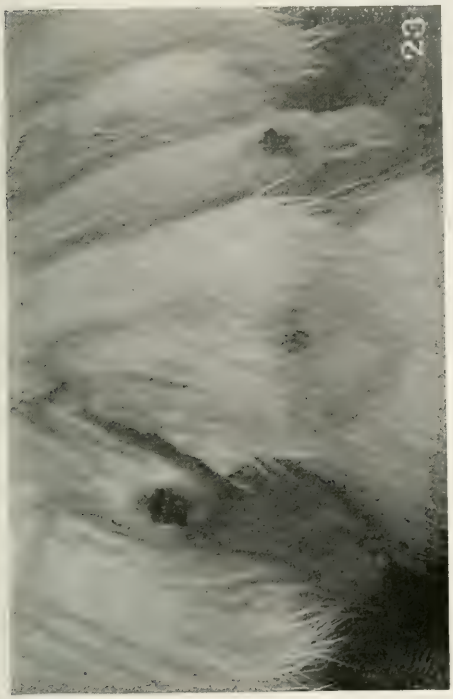
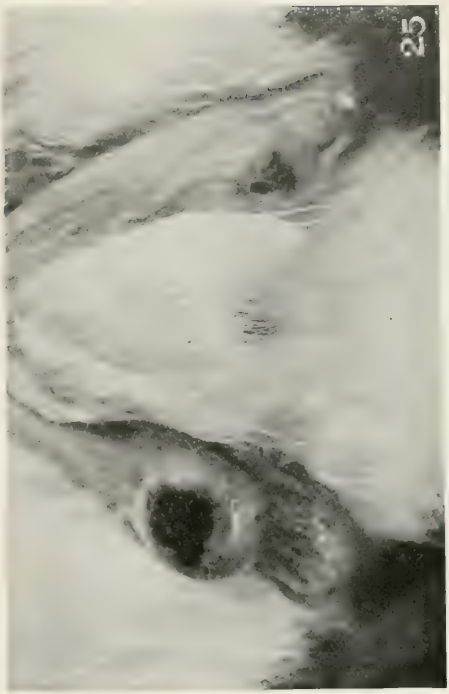
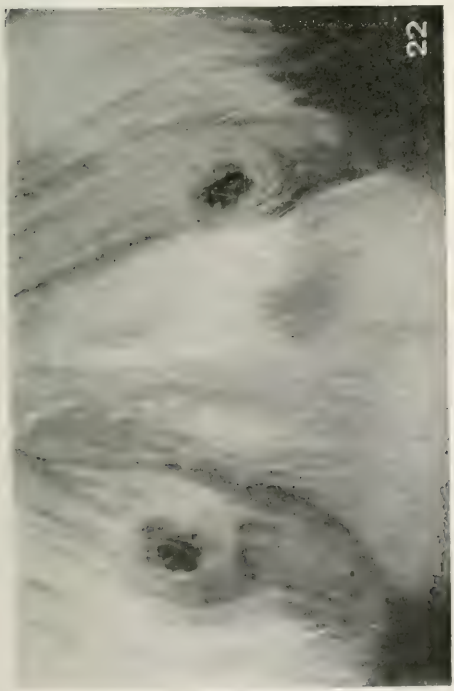


(Brown and Pearce: Trypanosome and spirochete infections.)

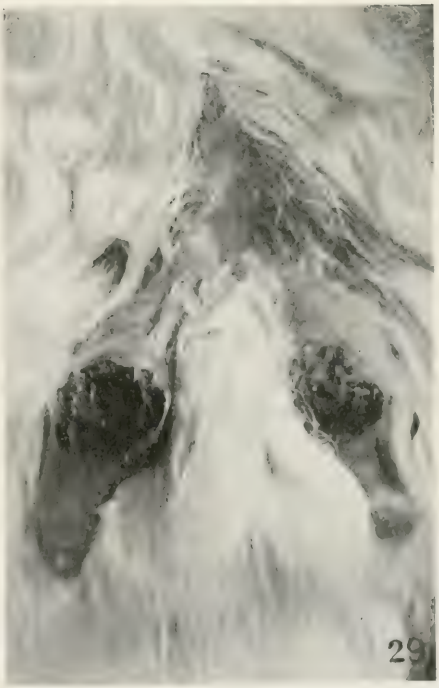
(Brown and Pearce: Trypanosome and spirochete infections.)







(Brown and Pearce: Trypanosome and spirochete infections.)



(Brown and Pearce: Trypanosome and spirochete infections.)

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THE INFLUENCE OF DEGENERATION OF ONE VAGUS NERVE UPON THE DEVELOPMENT OF PNEUMONIA.

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(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Read before the Academy, April 28, 1919.)

Since Galen's time experiments have been made upon the vagus nerve and it has been generally known that section of both nerves leads to an early death of the animal. It was however in the seventeenth century that it first became known, through the observations of Valsalva and Morgagni, that the section of the vagi causes lesions in the lungs, lesions which were designated by Vieussens as inflammation. Since this period numerous studies have been made regarding the nature of the influence of cutting of the vagi which leads to inflammation of the lungs, to pneumonia. Many investigators were of the opinion that the section of the nerves leads directly to changes in the pulmonary tissue. The last representative of this group of investigators was Schiff who in the forties of the last century described the lesion as being due to a *neuroparalytic* action. At the same period, however, Traube carried out many series of well conducted experiments by which he seemed to prove that the section of both vagi causes lesions in the lung by paralysis of the oesophagus, which prevents the transportation of food into the stomach, and by paralysis of the nerves of the larynx, a paralysis which facilitates the entrance of food, saliva, and other foreign bodies into the trachea and the lungs. In other words, the pneumonia following section of both vagi was not due to direct changes in the lung tissue but indirectly to the entrance of foreign bodies through the trachea into the lung; the inflammation following section of both vagi was of the nature of 'aspiration pneumonia.' In the last six or seven decades many more experiments have been made on the section of both vagi; but all investigators have

accepted Traube's interpretation, namely, that the section of the vagi does not cause directly any changes in the tissues, but that the section of the nerves leads to a 'foreign body pneumonia.'

In the above mentioned numerous experiments, as a rule, both vagi were cut; not very many experiments were made by cutting only one vagus. But when such an experiment was made, it was found practically invariably that cutting of one vagus produced neither any clinical effect during life nor were there pathological lesions of the lung at autopsies. I may add that our own experimental experience supports this finding: Cutting of one vagus of a normal dog never leads to a pulmonary disease or lesion.

In the course of the last eight years we carried out in this laboratory numerous experiments on the production of pneumonia by direct injection through the unoperated larynx into the bronchi of normal dogs of various micro-organisms capable of producing various forms of pneumonia. Most of our experiments were recently made with intrabronchial injections of culture of pneumococcus type I, and it was established that the degree of the disease which it produces or the length of time required for the fatal outcome depend upon the virulence of the organism as well as the quantity of the culture injected. Recently a long series of such experiments were carried out with a view of studying the *direct action of vagus nerves upon the development of pneumonia.* Since in these experiments the nerves of the oesophagus and of the larynx remained intact, any acceleration in the development of the disease or the pulmonary lesion could be ascribed only to the interruption of the nervous impulse normally transmitted through the intact vagus nerve to the lung tissue. We shall not enter here into the details of the experiments; but merely state briefly that: In one series of experiments one vagus was cut but no organisms insufflated; these animals, as mentioned above, remained normal. In another series of experiments a definite quantity of the culture was insufflated in normal dogs. These animals either remained alive or died with some pulmonary lesions many days after the injection. In a third series the same culture and the same quantity of it was insufflated on the same day and under the same condition as in the last mentioned series; but in these animals one vagus was cut either four days or ten or more days before the culture was insufflated. These experiments brought

to light a striking result. *In the series in which one vagus was cut ten or more days before the insufflation of the culture nearly all animals died in less than twenty-four hours after receiving a certain (minimum) amount of the culture*, while in the animals in which one vagus was cut four days before the injection or not cut at all, not a single animal died in such a short period, and most of them remained alive. In other words, a certain minimum quantity of culture which was injected ten or more days after one vagus was cut proved to be rapidly fatal; while the injection of the same quantity remained ineffective if no vagus was cut or was cut only a few days before the injection of the culture.

We shall confine ourselves here to the communication of these facts and to only one interpretation of them. First, these experiments show unmistakably that the integrity of a certain form of nerve impulses is indispensable for the normal resistance of the lung tissue to infection or intoxication; if one vagus nerve was cut ten days before the injection of a pneumococcus culture a certain mimimum of the culture was nearly invariably rapidly fatal, while in a normal animal or in an animal in which one vagus was cut only a few days before the injection or not cut at all, the same minimum of culture has little or no effect.

Second, for the vessels of most of the tissues it is known that they are innervated by vasoconstrictors and vasodilators. It is further known that the vasoconstrictors degenerate about four days after the section of the nerves, while the vasodilators degenerate only about ten days or later after the section. If we assume that the lungs are also provided with vasoconstrictors and vasodilators and that these vasomotor fibres are carried to the lungs in the vagus nerves, we would have a plausible explanation for the phenomena with which we were confronted in our experiments, namely, cutting of the vagi four days previous to the culture injection is capable of causing degeneration of only the vasoconstrictor nerve fibres, which may not be indispensable to the upholding of the normal activity of the lung tissue. If, however, the injection is made about ten days or longer after the cutting of one vagus, also the vasodilators are degenerated and their integrity may be indispensable for upholding the circulation and the normal resistance of the lung tissue to infection or intoxication by a virulent culture of pneumococcus type I.

At any rate, our experiments brought out the facts that nerve impulses are running through the vagus nerves which are important to the upholding of the normal resistance of the lung tissue itself. That even one vagus nerve is an important factor in this process; and that these activities of the vagus nerves can be recognized only after an intrabronchial or intratracheal injection of a virulent culture ten days after the cutting of one vagus.

Furthermore, the fact that the intrabronchial injection of a minimum culture proved fatal only when the injection was made ten days after the section of one vagus and the further consideration of the fact that it takes about ten days for the degeneration of vasodilators lend support to the assumption that it is the degeneration of the vasodilators which is responsible for the fatal results observed in our experiments.

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PFEIFFER'S BACILLUS AND INFLUENZA.

A Serological Study.

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The literature which has grown up in the very short time since the pandemic of influenza in 1918 is already so voluminous that it cannot be abstracted and analyzed in a brief space. Moreover, such an analysis, at the present time, would not be profitable. The methods employed by different bacteriologists in cultivating the influenza bacillus of Pfeiffer have been so diverse that comparison of results is valueless. There is, at present, hardly any difference of opinion that, with an adequate bacteriological technique, Pfeiffer's bacillus is found to be very commonly present in the respiratory tract of persons suffering from influenza and its attendant pneumonia. Moreover, the bacillus is also widespread in the upper respiratory mucous membranes of persons who have not had influenza, and even of persons who have not, so far as is known, been exposed to the epidemic disease.

We may accept the wide prevalence of the so called influenza bacillus as established and admitted and then proceed to the next and more important, because essential, question of the relationship of the bacillus to the symptom-complex influenza. Except in epidemic periods this symptom-complex is not so definite as to enable a sure and prompt diagnosis of influenza to be made; hence the cultivation of the Pfeiffer bacillus in interepidemic periods, from the upper respiratory tract, is not significant, necessarily, of clinical influenza. Possibly, indeed, there is no pathogenic microorganism, except the omnipresent pyogenic cocci, so frequently present in those parts as the Pfeiffer bacillus.

But even though the etiological rôle of the Pfeiffer bacillus in influenza has come to be seriously questioned, the part it plays in the

pathological complex characterizing this severe disease may still call for definition. Fortunately, in respect to this point there are other means available than mere presence in cultures to determine pathogenic action. The study on which this paper is based was begun early in the epidemic. In the meantime other publications bearing on the same topic have appeared. But it would seem that the subject is one on which additional evidence and more varied tests are desirable; hence the presentation of our results.

Culture Media.

Rabbit blood agar was the routine medium used in searching for Pfeiffer's bacillus during life and at autopsy, until Avery's oleate agar[1] became available. For serological work a medium made in the following way proved most suitable. Fresh rabbit blood was boiled for 2 minutes in a water bath, then centrifuged. The resulting clear, pale pink or yellow fluid was found by spectroscopic examination to contain hemoglobin. Two or three drops were added to 5 cc. of melted agar (pH 7.5) and slanted, while 0.5 cc. was added to a tube containing 20 cc. of broth (pH 7.8). Thus a solid medium and a fluid medium were obtained in which Pfeiffer's bacilli grew abundantly, and washing to get rid of blood or serum was not necessary. The bacilli live only 5 to 6 days on this medium, however. To keep stock cultures, blood broth, in which the bacilli remain viable for 6 weeks or more, proved by far the most suitable medium, while for isolation of the organisms the oleate agar was best.

Serological Reactions.

It was clear that for serological study sera from convalescent patients as well as monovalent immune sera experimentally produced were needed. Rabbits weighing 1,600 gm. proved to be best suited for the purpose of immunization. By means of intravenous injections of live Pfeiffer bacilli, in increasing doses, administered every 3rd day, antisera were produced with ten strains, seven isolated during the epidemic and three obtained several years ago.

[1] Avery, O. T., *J. Am. Med. Assn.*, 1918, lxxi, 2050.

Agglutination.—It was difficult to get even suspensions of Pfeiffer's bacillus suitable for agglutination tests. Blood broth cultures were not sufficiently profuse, and cultures grown on solid medium clumped spontaneously whether washed or not. Centrifuging had the effect of making the bacilli stick together in small clumps which could not be broken up. Finally, bacilli grown on the boiled-blood-centrifugate-agar were suspended in distilled water, while the serum dilutions were made in normal salt solution. The mixtures were kept at 55°C. for 2 hours, then at room temperature over night. At the end of 2 hours no agglutination had taken place; consequently the 18 to 20 hour reading was the only one that could be noted. The controls obtained by this method were fair for most strains, but unsatisfactory for others. Three strains clumped so much spontaneously that they could not be used for agglutination tests. The strains of Pfeiffer's bacillus varied in the degree of their agglutination reactions, as well as in the kind; four were inagglutinable in every serum tested, one agglutinated only in its homologous monovalent rabbit serum in dilutions of 1: 100, while three other strains clumped in 1: 50 dilutions in that serum. The presence of higher agglutinin content for heterologous than for homologous strains was noted for three sera made with strains of Pfeiffer's bacilli isolated from epidemic cases.

One normal rabbit serum was encountered which agglutinated the Pfeiffer bacillus in a dilution of 1: 20.

The serum of five normal human adults did not agglutinate Pfeiffer's bacillus in any dilution, while two other normal sera reacted in dilutions not exceeding 1: 10. Sera from eleven patients were tested during the 2nd week of illness. Five had had a mild bronchopneumonia attending the influenza attack, and the serum from two of these agglutinated Pfeiffer's bacilli in dilutions of 1: 100, while the other three sera did not contain agglutinins for these bacilli in dilutions higher than 1: 40. Sera from six cases of simple influenza without pneumonia contained no agglutinins for Pfeiffer's bacillus in two instances, and reacted in dilutions of 1: 40 in four others.

Agglutination reactions with the bacillus of Pfeiffer were not satisfactory because of the tendency to spontaneous clumping of the organisms. The wide variations in the results recorded in the litera-

ture may depend partly on this difficulty. Fleming[2] studied twenty-one influenza patients and found the agglutinin content of their sera low; only six agglutinated Pfeiffer's bacillus in dilutions higher than 1:32, and of these only one reacted above 1:128, giving a positive reaction in a dilution of 1:1,000. He also found that strains differed in their ability to agglutinate.

Adults who had been inoculated with vaccine made from several strains of Pfeiffer's bacilli developed agglutinins in their blood within 6 to 10 days, but only rarely were these present in dilutions higher than 1:100.

Complement Deviation.—Antigens were made in two ways: (*a*) Cultures grown in blood broth were centrifuged to throw out the blood cells, and the supernatant fluid was pipetted off, heated to 55°C. for 45 minutes, and tested. Against this antigen controls with sterile broth were always done in parallel series.

(*b*) Cultures in boiled-blood-coagulate-broth were centrifuged for 30 minutes and the precipitate was washed twice in salt solution, then resuspended in fresh salt solution and tested. The use of tricresol was discarded because it tended to make the antigens anticomplementary. A standard of turbidity for the suspended washed bacilli was evolved which approximated 10 cc. of salt solution to the precipitate from 25 cc. of broth culture. However, this amount had to be adjusted to the individual growth.

Tests with cultures of varying ages showed that the maximum strength of antigen was obtained in 3 days. The strains of Pfeiffer's bacilli varied in their antigenic power. In seven instances the antigen was strong, in eight, weak; while seven other strains failed to yield an active antigenic product. Whenever possible, sera were tested against antigen made from (*a*) a meningeal strain isolated during the epidemic, and (*b*) another meningeal strain isolated 2 years ago; (*c*) three respiratory epidemic strains; (*d*) a sporadic respiratory strain; (*e*) a strain isolated from a healthy carrier. Blood from forty-four persons was tested.[3] Of these, fourteen were patients convalescent 6 to 23 days after the onset of an attack of influenza, nineteen

[2] Fleming, A., *Lancet*, 1919, i, 138.

[3] I am indebted to Dr. Walter W. Palmer for his courtesy in allowing me to obtain blood from several patients in the wards of the Presbyterian Hospital.

had recovered from influenza 1 to 4 months previously, four were healthy carriers who had never been ill with influenza, and four were healthy controls. One fatal case of influenzal meningitis was studied, and also two patients who had suffered from diseases other than influenza—lobar pneumonia in one instance, laryngitis and pharyngitis in the other.

Sera from four normal adults were tested as controls. They had not been ill during the past fall and winter, had not suffered from colds, had not been vaccinated against influenza, and carried no Pfeiffer's bacilli in their sputum. No complement-binding reaction was obtained with any antigen in any of these four sera.

Sera from convalescent patients were examined at the end of the 1st week and reexamined in three cases at the end of the 2nd week after onset. Dilutions of 1:5, 1:10, and 1:20 were used and showed a diminishing strength of reaction. The results are shown in Table I. All the sera gave a fixation reaction on the 6th or 7th day, and all but two reacted with antigens made from more than one strain of Pfeiffer's bacillus. The three sera which were obtainable a second time reacted more strongly at the end of the 2nd week than on the 6th day. Five were mild cases without any signs of broncho-pneumonia, while seven were complicated by pneumonia. It is to be noted that the sera of patients who suffered from pneumonia complicating influenza had stronger complement-binding power than did the sera of uncomplicated cases. No convalescent case failed to show fixation antibodies at the end of a week, though these were sometimes small in amount and limited in kind. No differentiation of strains was possible from these results.

One patient had been inoculated 5 weeks before she became ill with influenza with a vaccine of Pfeiffer's bacillus only. Her serum gave a reaction with but one antigen of five used. The influenzal attack had been a mild one, and apparently the inoculation did not increase the complement-binding power of her serum in the presence of Pfeiffer bacillus antigen.

An infant with Pfeiffer bacillus (influenzal) meningitis, who died on the 7th day of illness, gave a strongly positive reaction on the 6th day, with two antigens, one made from a meningeal strain of Pfeiffer's bacillus isolated 2 years before, and the other made from a strain

TABLE I.

Complement Fixation with Blood of Convalescent Patients.

Individual No.	Vaccine.	Day of illness.	Serum dilution.	Antigens.							
				M	D	325	R	W	T	I	280
1	1 mo. before.	9th	1: 5	0	+++	0		0			0
			1:10	0	+++	0		0			0
			1:20	0	+	0		0			0
2	0	9th	1: 5	+++	+++	+++		+++			0
			1:10	+++	+++	++		++			0
			1:20	+	++	+		0			0
3	0	7th	1: 5	0	+	++	++	++	++	++	++
			1:10	0	+	++	+	+	++	+	0
			1:20	0	0	+	0	0	0	0	0
	0	13th	1: 5	+	+++	+++	+++	+++	+++	+++	++
			1:10	0	+++	+++	+	++	++	+	0
			1:20	0	++	++	+	+	0	0	0
4	0	6th	1: 5	0	+	0	0	0		0	0
			1:10	0	+	0	0	0		0	0
			1:20	0	0	0	0	0		0	0
	0	12th	1: 5	0	+++	0	0	0		0	0
			1:10	0	+++	0	0	0		0	0
			1:20	0	++	0	0	0		0	0
5	0	6th	1: 5	+	++	0		0		0	0
			1:10	0	+	0		0		0	0
			1:20	0	0	0		0		0	0

W is a strain of Pfeiffer's bacillus isolated from a case of influenzal meningitis during the epidemic; T a strain isolated from a case of influenzal meningitis 2 years ago; M and D strains isolated from the lungs of influenzal pneumonia cases during the epidemic; 325 and R strains isolated from the sputum of influenzal pneumonia cases during the epidemic; I a strain isolated from the lungs of an influenzal pneumonia case 3 years ago; and 280 a strain isolated from the throat of a healthy adult carrier.

0 indicates complete hemolysis, no fixation of complement; + + + no hemolysis, complete fixation of complement; + + and + varying degrees of fixation of complement.

TABLE I—*Concluded.*

Individual No.	Vaccine.	Day of illness.	Serum dilution.	Antigens.							
				M	D	325	R	W	T	I	280
5	0	12th	1: 5	++	+++	0		0		0	0
			1:10	0	+++	0		0		0	0
			1:20	0	+	0		0		0	0
6	0	7th	1: 5	0	+++	++	+++	+++			
			1:10	0	+++	++	+++	+			
			1:20	0	++	0	+	+			
7	0	8th	1: 5	0	+++	+++	+++	+++			
			1:10	0	+++	++	+++	+			
			1:20	0	+++	0	+	0			
8	0	14th	1: 5	0	+++	+++	0	+++			
			1:10	0	+++	+	0	+			
			1:20	0	+	0	0	+			
9	0	6th	1: 5			+++			++		
			1:10			+++			++		
			1:20			+			+		
10	0	9th	1: 5	+	+++	++	++	+++	+++	++	++
			1:10	0	+++	++	+	+++	+	+	+
			1:20	0	+	0	0	++	0	0	0
11	0	7th	1: 5		+++					+	
			1:10		+					+	
			1:20		0					0	
12	0	14th	1: 5	++	+++				++	++	
			1:10	++	+++				+	+	
			1:20	0	+++				0	+	
13	0	13th	1: 5		+++					++	
			1:10		+++					+	
			1:20		++					+	
14	0	23rd	1: 5	+++	+++	+++	++		++	++	++
			1:10	++	+++	+++	+		++	++	+
			1:20	+	+	++	+		++	++	+

recovered from the lung in a fatal case of bronchopneumonia occurring during the epidemic. With antigen of a strain of Pfeiffer's bacillus from a healthy carrier, isolated during the course of the epidemic, and with that of a respiratory strain isolated from a sporadic case 4 years ago, no reactions were obtained. It is to be regretted that it was not possible to obtain a larger quantity of serum from this baby, so that it might have been tested with antigen made from its homologous strain of Pfeiffer's bacillus. The results obtained with this serum confirm the point previously shown;[4] namely, that meningeal strains of Pfeiffer's bacillus are virulent and yield a strong antigen, and that while respiratory strains are more apt to be nonvirulent and yield a weak antigen, virulent respiratory strains may be encountered which yield a strong antigen.

Complement fixation tests with Pfeiffer bacillus antigen made with the blood of nineteen individuals who had entirely recovered from influenza, which had attacked them 1 to 4 months previously, gave irregular results. At the end of a month one serum gave only an incomplete reaction with two antigens, and after 6 and 7 weeks only low complement-binding content was found in two other sera. 2 months after the illness no reaction was obtained with one serum, while three others gave strong reactions even in dilutions of 1:20, and a fourth reacted well in a dilution of 1:5, but not higher. After 3 months one serum gave complete reactions only in dilutions of 1:5, while another was positive in dilutions of 1:10. 4 months after influenza two sera were entirely devoid of complement-binding body content.

1 month after the influenzal illness a man who had been inoculated with three doses of vaccine made with three strains of Pfeiffer's bacillus, 4 months before the attack began, gave very strong reactions in dilutions of 1:10. 2 months after the attack of influenza the sera of two subjects inoculated with Pfeiffer bacillus vaccine 3 months before the illness reacted as strongly as did two unvaccinated patients, while one reacted much less well. In comparing the serum of inoculated and of non-inoculated individuals, it becomes evident that the administration of a vaccine, made from several strains of Pfeiffer's

[4] Wollstein, M., *J. Exp. Med.*, 1915, xxii, 445.

bacillus, did not have any apparent influence on the degree of the complement-binding reaction with the antigens of Pfeiffer's bacillus when an interval of 3 or 4 months had elapsed between the inoculation and the influenzal illness. The patient whose serum was poor in this fixing antibody 1 month after the attack is apparently the exception, when viewed in the light of Table I.

Sera from four carriers were tested. All denied any respiratory illness in the previous 6 months. One had been inoculated with vaccine made from Pfeiffer's bacilli only. Two of the four carriers gave no reaction with several antigens. The inoculated individual and one person who had not been vaccinated reacted strongly. One of the carriers whose serum was negative to this test received Pfeiffer bacillus vaccine, and 1 week after the third dose had been injected his serum contained complement-binding bodies for three of six antigens against which it was tested.

To sum up, complement-binding antibodies were absent from the blood of four normal individuals who had not had influenza, and who were not carriers of Pfeiffer's bacillus. In the blood of influenza patients the fixing antibodies were present at the end of the 1st week, increasing in strength during the 2nd week, and were, as a rule, demonstrable at the end of 3 or 4 weeks. At the end of the 2nd month after an attack of influenza these antibodies were strongly present except in one case. At the end of the 3rd month complement fixation was still demonstrable in patients' serum, but in 4 months it had entirely disappeared. A complicating pneumonia increased the complement-binding power of the serum.

Monovalent immune rabbit sera were made with ten strains of Pfeiffer's bacillus and all bound complement in the presence of antigens made both from homologous and from heterologous strains. Normal rabbits were used as controls and their sera gave negative results.

Precipitins.—With the same antigen as that used for the complement fixation test, precipitins were demonstrable in every convalescent patient's serum tested, while they were absent from the serum of normal persons. Antigens made from homologous strains of Pfeiffer's bacillus gave no stronger precipitin reactions than did those made from heterologous strains. The earliest precipitin reaction noted was on the 6th day of the illness, and the latest, 3 months after the attack.

Poisonous Filtrates.

According to Parker[5] filtrates of Pfeiffer bacillus cultures grown in heated blood broth for 6 to 20 hours are poisonous for rabbits when inoculated intravenously. Tests were made with twenty-five strains of the bacilli. The filtrates from seven strains killed rabbits weighing 1,300 to 1,600 gm. within 1 to 2½ hours when inoculated intravenously in doses of 2 or 3 cc. Much larger doses (5 cc.) were required to kill the animals with filtrates of other strains. Death was sometimes, though rarely, delayed until 12 to 15 hours after the injection of the poison.

The effects of intravenous injection of a potent filtrate from an early culture of Pfeiffer's bacillus were quite uniform. The animals gradually became more and more quiet, so that in 30 to 45 minutes they sat in a hunched up position with no movement but that of very rapid respiration. Intestinal peristalsis was increased until at the end of 60 to 90 minutes the animals were passing fluid feces. Gradually the head leaned, then fell to one side, and the animal slowly fell over. After one or two attempts to rise, all muscular efforts ceased, and it lay on its side, breathing very rapidly and irregularly, with the head becoming more and more retracted. Death was often preceded by general convulsions. Non-lethal doses caused the symptoms of muscular weakness, irregular respiration, retraction of the head, and diarrhea; but the rabbit did not fall over on its side or go into a convulsion, and recovery gradually took place so that the animal was well within 3 or 4 hours, though more quiet than before the injection. The leucocytes fell within 1 hour after inoculation, and a difference of 5,000 to 12,000 from the initial count was noted. The recovered animals showed a rise within 4 to 5 hours, and on the following day the leucocytes were always as high or higher than before the injection of the filtrate.

Postmortem examination showed constant changes in the lungs. These organs were deep pink in color, mottled with dark red or brown areas of hemorrhage, often most marked in one or both lower lobes. Punctate subpleural hemorrhages were numerous and general. The lungs were edematous; frothy fluid exuded from the cut surface and

[5] Parker, J. T., *J. Am. Med. Assn.*, 1919, lxxii, 476.

from the trachea and bronchi. The mucosa lining the larynx, trachea, and large bronchi was dark red or purple in color from an intense congestion, while bright red points of capillary hemorrhages were numerous.

The intestinal contents were fluid. The kidneys and liver were congested. In female rabbits the Fallopian tubes were always bluish red in color and on section showed an intense congestion of all the coats. The blood vessels in the cerebrospinal meninges were filled with blood. No gross hemorrhages were apparent in the nervous system. The suprarenals were pale pink in color.

Microscopic examination of the brain showed minute areas of perivascular diapedesis in sections of the medulla. The lungs showed a congestion of all the vessels so exquisite as to constitute a natural injection. A comparative absence of leucocytes was striking; both within the lumen of the vessels and in the alveoli they were few in number. The connective tissue septa were edematous. Many alveoli contained red blood cells, and the epithelium lining the walls was intact; other alveoli contained granular, coagulated serum in which air bubbles were apparent.

In the female the Fallopian tubes showed an intense congestion of the blood vessels in the mucosa, while some muscle fibers had undergone hyaline degeneration.

Control experiments were made by injecting normal incubated broth intravenously into rabbits. No symptoms followed, and at autopsy no lesions were found. A filtrate of typhoid bacillus culture was also injected as control. The animals died after 15 to 18 hours, and at autopsy showed a hypostatic congestion of the lungs and some discoloration due in large part to the fact that they had been dead several hours before they were examined. The edema and punctate hemorrhages were lacking.

The sera of rabbits inoculated with increasing doses of filtrates of young cultures of Pfeiffer's bacilli occasionally protected other rabbits against one fatal dose of homologous and heterologous filtrates, but no serum protected against more than one lethal dose of poison. Normal rabbit serum protected almost as many animals as did the sera of the inoculated rabbits. The nature of the poison remains in doubt, but it is evidently not a toxin, since there is no definite incu-

bation period between its inoculation and the appearance of symptoms, and a protective serum does not result from its repeated injection into rabbits.

Protection Experiments.

Attempts to protect mice against lethal doses of Pfeiffer's bacilli by means of rabbit sera made with poisonous filtrates proved unsatisfactory. The sera never reached a high potency because the rabbits became emaciated and died when the injected poison was a strong one, and they reacted but slightly to a weak one. Consequently the results of protection experiments on mice with such a serum were irregular and misleading. A strong antibacterial serum was more readily obtained and protected a greater number of mice against a lethal dose of Pfeiffer's bacilli, whether the serum was given 24 hours before the bacilli were injected, or after 15 minutes contact with the dose of culture (Table II).

TABLE II.

Protection Experiments with Mice.

No. of animals.	Serum (rabbit).	Method of administration.	Survived.
			per cent
53	Antitoxic (?).	24 hrs. before bacilli.	3.8
34	" (?).	After 15 min. contact with bacilli.	23.0
16	Antibacterial.	" 15 " " " "	25.0
20	"	24 hrs. before bacilli.	40.0
10	Normal.	24 " " "	1.0
10	"	After 15 min. contact with bacilli.	2.0

Human sera from six recovered patients were entirely lacking in protective effect when tested on mice inoculated with lethal doses of Pfeiffer's bacillus, although all these sera contained complement-binding bodies.

DISCUSSION.

As regards the serological reactions of Pfeiffer's bacillus with the sera of recovered patients, as well as with monovalent immune rabbit sera, the results with agglutination were irregular and not satisfactory.

As far as they showed positive results the epidemic and the sporadic strains reacted similarly.

At the Department of Health of the City of New York, Park and his coworkers[6] found Pfeiffer's bacillus present in 80 to 100 per cent of influenza patients. The bacilli isolated from different cases, however, did not produce identical immune bodies in inoculated animals as measured by the agglutinin absorption test, and Park concludes from these studies that there were many strains of Pfeiffer's bacillus, and not one epidemic strain, present during the pandemic.

Complement fixation reactions with human sera showed that normal controls did not contain complement-binding bodies for the Pfeiffer bacillus, but that the blood of recovered patients gave a positive reaction with more or less regularity in dilutions varying from 1:5 to 1:20. The binding could almost always be obtained with antigens made from more than one strain of the bacillus. On the other hand, with the sera of two patients in whose sputum Pfeiffer's bacilli were not found, and who suffered from lobar pneumonia and laryngitis respectively, no fixation was obtained. It would seem, then, that the reaction is due to something more than the presence of an increased amount of non-specific protein after a febrile attack.

With some immune rabbit sera binding was obtained in dilutions of 1:100. Normal human and rabbit serum controls did not bind.

Rapoport[7] examined a much larger series of cases than I was able to do. He studied influenzal pneumonia patients and controls. His results showed complement fixation bodies in 54.5 per cent of 295 convalescent influenzal pneumonia patients and in only 9.67 per cent of 300 controls. My results confirm his and show that patients who are convalescent from influenza without pneumonia also may have complement fixation bodies in their serum for a limited period of time and to a less marked degree.

Kolmer, Trist, and Yagle[8] demonstrated complement fixation in the serum of convalescent influenza patients with antigens made from Pfeiffer's bacillus, hemolytic streptococci, and *Micrococcus catarrhalis* but not with staphylococcus and pseudodiphtheria bacillus antigens.

[6] Park, W. H., *J. Am. Med. Assn.*, 1919, lxxiii, 318.
[7] Rapoport, F. H., *J. Am. Med. Assn.*, 1919, lxxii, 633.
[8] Kolmer, J. A., Trist, M. E., and Yagle, E., *J. Infect. Dis.*, 1919, xxiv, 583.

45 to 50 per cent of the sera reacted with Pfeiffer's bacillus, and only 38 per cent bound complement when streptococcus or *catarrhalis* antigens were employed. All the sera were tested in a dilution of 1:10. Consequently no light is thrown on the specificity of the reactions.

Precipitins were also found constantly in the sera of recovered patients and of immunized rabbits. The reaction for them was strongly marked both with heterologous and with homologous strains of Pfeiffer's bacillus.

CONCLUSIONS.

In has been shown that the sera of patients convalescent from influenza yield reactions for agglutinins, precipitins, and complement-binding bodies with antigens of Pfeiffer's bacillus. These reactions appear constantly at the end of the 1st week, increase in intensity during the 2nd week, and remain demonstrable for a peried of 2 to 4 months. They were most complete in the sera of patients suffering from postinfluenzal pneumonia. It has also been demonstrated that the strains of Pfeiffer's bacillus isolated during the epidemic were morphologically and biologically similar to the strains isolated from influenza cases in other years, and antigenically they differed from them only quantitatively. The patients' serological reactions indicate the parasitic nature of the bacillus, but are not sufficiently stable and clean-cut to signify that Pfeiffer's bacillus is the specific inciting agent of epidemic influenza. They do, however, indicate that the bacillus of Pfeiffer is at least a very common secondary invader in influenza, and that its presence influences the course of the pathological process.

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THE ACTION OF INTRAVENOUS INJECTIONS OF PANCREAS EMULSIONS IN EXPERIMENTAL DIABETES.

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It is evident that the demonstration of a beneficial effect of a pancreas preparation, when administered parenterally to a diabetic animal, would be of importance both theoretically and practically. Theoretically it would support the internal secretion hypothesis of the origin of diabetes. Practically it would suggest a possible therapeutic application.

The early work in this field was either negative or, as Pflüger,[1] and Leschke[2] have shown, is open to serious criticism. The usual criterion was a lowering of the concentration of sugar in the urine or a diminution of the total 24 hour output of sugar. Either of these effects may easily be caused by a diminished intake of food, due to the loss of appetite usually resulting from the treatment, to a change in the character of the diet, or to an influence on the kidneys. In clinical experience, the *feeding* of pancreas preparations has sometimes seemed to have a favorable subjective effect but with no constant antidiabetic action. Often this appeared to be due to supplying the *external* secretion to patients in need of it. Parenteral administration usually has not given good results. In a brief résumé of the literature Allen[3] says: "Though pancreas feeding may have at least a digestive value in some cases of diabetes, injections of pancreatic preparations have proved both useless and harmful. The failure began with Minkowski, and has continued to the present without an exception."

The more recent work may be summarized very briefly. Scott[4] found that intravenous injections of water extracts of pancreatic tissue, which had previously been extracted with alcohol, diminished temporarily the sugar excretion

[1] Pflüger, E., *Arch. ges. Physiol.*, 1907, cxviii, 267.

[2] Leschke, E., *Arch. Physiol.*, 1910, 401.

[3] Allen, F. M., Studies concerning glycosuria and diabetes, Cambridge, 1913, 815.

[4] Scott, E. L., *Am. J. Physiol.*, 1911–12, xxix, 306.

of diabetic dogs and lowered their D/N ratio. Murlin and Kramer[5] injected boiled pancreatic extracts into depancreatized dogs and found a temporary reduction in the output of sugar. A mixed boiled extract of pancreas and duodenal mucosa produced a greater fall, and in one case a complete disappearance of the urinary sugar. The results were referred by these authors mainly to the sodium carbonate present in the extracts, because Ringer's solution, which had been brought to about the same degree of alkalinity as the medium used for the extract, was found to have similar effects. Later the preliminary announcement[6] of the present work led Murlin and Kramer[7] to try unboiled pancreatic extract. This was given by mouth, however, and was mixed with Na_2CO_3. A favorable influence on the respiratory quotient was observed in two experiments.

The present work was undertaken because of the remarkable influence which an emulsion of pancreas had been found to exert upon the disposition of intravenously injected dextrose in diabetic dogs. In normal animals[8] large quantities of sugar, introduced intravenously, were promptly lost from the circulation; in diabetic animals,[6,9] on the other hand, this occurred very slowly, as was shown by the fact that even an hour and a half after the end of the sugar infusion the blood sugar was still far above its original level. Now, when an emulsion of pancreas was mixed with the glucose solution to be injected, the diabetic animal handled the sugar in nearly a normal manner.[6] Then the question naturally arose whether a similar pancreatic emulsion would not help the diabetic organism to dispose of its own excess sugar also.

In testing this question there were two principles which, we felt, should be followed. First, since the chemical properties of the effective substance or substances in the pancreas were unknown, it seemed necessary to avoid complicated procedures of purification; therefore, a simple water extraction with subsequent dilution with saline was adopted. Secondly, since depancreatization brings on its effects within a very short time, it appeared that normally

[5] Murlin, J. R., and Kramer, B., *J. Biol. Chem.*, 1913, xv, 365.

[6] Kleiner, I. S., and Meltzer, S. J., *Proc. Nat. Acad. Sc.*, 1915, i, 338.

[7] Murlin, J. R., and Kramer, B., *J. Biol. Chem.*, 1916, xxvii, 517.

[8] Kleiner, I. S., and Meltzer, S. J., *Am. J. Physiol.*, 1914, xxxiii, p. xvii. Kleiner, I. S., *J. Exp. Med.*, 1916, xxiii, 507.

[9] Kleiner, I. S., and Meltzer, S. J., *Proc. Soc. Exp. Biol. and Med.*, 1914–15, xii, 58.

the pancreas secretes its effective substance into the blood stream continually in very small amounts. It was therefore decided to introduce the pancreatic preparation by the intravenous route very slowly and over an appreciable period; the results, however, might be expected to be observed during the injection and for only a few hours thereafter. These principles were followed and the results were very gratifying—in a word, the slow intravenous infusion of an aqueous pancreatic "emulsion" usually resulted in a temporary but marked decrease in the glycemia and glycosuria. The first few experiments were briefly reported in 1915.[6] The present paper includes these experiments as well as a large number of experiments performed since the first communication.

Method.

Operation.—Total depancreatization was performed in the majority of cases. In three animals, used in the six most recent experiments, the "Allen" procedure, *i.e.* leaving a small remnant around the large pancreatic duct, was adopted. The operations were performed under ether anesthesia.

Preparation of "Emulsion."—Fresh dog's pancreas was hashed, mixed with three or four times its weight of sterile distilled water, and placed in the refrigerator. After a period of from 1 to 20 hours it was strained and squeezed through muslin. The fluid thus obtained was diluted with 5 volumes of sterile 0.9 per cent NaCl solution before injection. This dilute solution was faintly acid or neutral to litmus, light pink in color, and almost clear. At no stage was it filtered, nor was sodium carbonate or any other substance added. In one experiment (LP77a), Ringer's solution was used in the extraction and also in the dilution; this did not lead to a better result. The emulsions of other tissues, used in the control experiments, were prepared in a similar manner.

Injection and Blood Sampling.—Some time before the infusion was to be given, the animals (in most of the experiments) received a small dose of morphine sulfate subcutaneously, usually about 1.5 mg. per kilo. Cannulas were then introduced under local anesthesia into a convenient vein and artery, for injection and blood sampling,

respectively. In the six recent experiments no morphine was given and only one cannula (the venous) was introduced under local anesthesia (ethyl chloride). In this series the blood was drawn directly from an external jugular vein into a syringe. The urine was obtained by catheter in all cases.

Analytical Methods.—The blood sugar determinations were made by the Myers-Bailey[10] modification of the Lewis-Benedict method. Glucose in the urine was estimated by a modification of the Pavy[11] method or by Benedict's[12] method. Hemoglobin was determined in most of the experiments by discharging 2 cc. of blood into dilute HCl (about 0.3 per cent) in a 250 cc. volumetric flask, diluting to this volume, and comparing the density of the color in a Duboscq colorimeter. The first blood sample of each experiment was used as the standard and was taken as 100 per cent for that experiment. Later the Sahli hemoglobinometer was used and, to avoid confusion, the results are similarly expressed.

Four illustrative protocols, including three injections of pancreas emulsion and one control, follow.

Experiment LP72a.—From Table I it will be observed that the high blood sugar value of 0.31 per cent fell to 0.14 per cent in the course of the 78 minute injection period and after another period of 90 minutes it was still low (0.13 per cent). Both of these figures, 0.14 and 0.13 per cent, are practically normal values, but emphasis is to be placed on the extent of the drop, from 0.31 to 0.13 per cent, a fall of 0.18 per cent, rather than upon the absolute value finally obtained. At the same time this decrease in the concentration of the blood sugar is not accompanied by any change in the hemoglobin value, or by an increased glycosuria. In other words it is not due to a dilution of the blood sugar by the injection fluid, or to a washing out of the sugar through the kidneys. In fact the kidney secretion of sugar is greatly diminished.

This was one of the few experiments in which the injection produced any noticeable general symptoms; there was first a fall and then a rise in temperature. There was no marked harmful effect

[10] Myers, V. C., and Bailey, C. V., *J. Biol. Chem.*, 1916, xxiv, 147.

[11] To be published shortly.

[12] Benedict, S. R., *J. Biol. Chem.*, 1911, ix, 57.

TABLE I.

Experiment LP72a.

Male, 11.25 Kilos, Completely Depancreatized April 19, 1915.

Date.	Time.		Blood sugar.	Hemoglobin in per cent of first sample.	Urine.			Temperature.
					Volume.	Glucose.		
	a.m.		*per cent*		*cc.*	*per cent*	*gm. per hr.*	°C.
Apr. 20	10.00	Injected 1.5 mg. of morphine sulfate per kilo subcutaneously.						
	10.23							39.1
	10.36	Cannulas have been introduced into left external jugular vein and right carotid artery under ethyl chloride and cocaine hydrochloride.						
	11.08				20	10.08		
	11.11		0.31	100				
	11.15–12.33	Injected intravenously 112 cc. of dilute pancreas emulsion prepared from the pancreas of this dog.						
	11.23	Cheyne-Stokes respiration during most of injection.						
	p.m.							
	12.34		0.14	101				37.9
	12.44				31.5	8.79	1.85	
	12.48							38.8
	1.00	Slight tremors of muscles of head and hind legs.						
	1.18							40.3
	1.35	Tremors have stopped.						
	2.04		0.13	100				
	2.15				42.5	1.62	0.46	
	2.18							40.8
	a.m.							
Apr. 21	9.45	Not catheterized.			193	1.49		39.3

of the infusion, however, as the animal was given a second infusion 2 days after the first and lived 2 days after that. The second infusion caused only a slight fall in the glycemia (see LP72b in Table V).

TABLE II.

Experiment LP76.

Female, 9 Kilos, Completely Depancreatized May 11, 1915.

Date.	Time.		Blood sugar.	Hemoglobin in per cent of first sample.	Urine.			Temperature.
					Volume.	Glucose.		
	a.m.		*per cent*		*cc.*	*per cent*	*gm. per hr.*	°C.
May 12	10.15							38.5
	10.40	Injected 1.4 mg. of morphine sulfate per kilo subcutaneously.						
	11.06	Cannulas have been introduced into left external jugular vein and left carotid artery under ethyl chloride and cocaine hydrochloride.						
	11.07	Catheterized.			111	+++		
	11.20							38.0
	11.43				20.5	11.16	3.82	
	11.45		0.33	100				
	11.49–12.52	Injected intravenously 112 cc. dilute pancreas emulsion prepared from the pancreas of this dog.						
	p.m.							
	12.53				30	12.38	3.18	
	12.54		0.25	101				
	1.13							39.0
	2.27				14	3.64	0.33	
	2.29		0.23	103				
	2.43							39.5

Experiment LP76.—In Experiment LP76 (Table II), in spite of an intense hyperglycemia and glycosuria the administration of pan-

creatic emulsion reduced the blood sugar and urine sugar to a marked degree, (0.10 per cent), without producing any unfavorable symptoms or affecting the temperature very much. 2 days later a differ-

TABLE III.

Experiment DP1a.

Female, 8.4 Kilos, 92 Per Cent of the Pancreas Removed on April 7, 1919, the Remnant Left Being in Communication with the Large Duct.

Date.	Time.		Blood sugar.	Hemoglobin in per cent of first sample.	Urine.			Temperature.
					Volume.	Glucose.		
	a.m.		*per cent*		*cc.*	*per cent*	*gm. per hr.*	*°C.*
Apr. 8	10.20		0.20	100				
	10.36	Catheterized				1.25		
	11.19							38.8
	11.30	Under ethyl chloride a cannula has been inserted in the right external jugular vein.						
	11.33		0.21	102				
	11.43				16	8.0	1.15	
	11.49–12.31	Injected intravenously 60 cc. of dilute pancreas emulsion prepared from the pancreas of this dog.						
	p.m.							
	12.36		0.12	102				
	12.44				10	5.40	0.53	
	2.15		0.08	96				
	2.23				14	0.5	0.04	
	2.26							40.4
	a.m.							
Apr. 9	10.08		0.27	99	800	0.33	0.20	
	10.20	Catheterized.			20	3.62		
	10.26							38.5

ent type of experiment was performed on this animal, after which it was killed with chloroform.

Experiment DP1a.—Experiment DP1a (Table III) was one of the more recent ones. No morphine was used and only an injection cannula was introduced into a vein. The animal was kept on the board only during the necessary experimental procedures. The

TABLE IV.

Experiment LP81a.

Male, 14.25 Kilos, Depancreatized June 7, 1915, at least 94 Per Cent of the Pancreas Removed.

Date.	Time.		Blood sugar.	Hemoglobin in per cent of first sample.	Urine.			Temperature.
					Volume.	Glucose.		
	a.m.		*per cent*		*cc.*	*per cent*	*gm. per hr.*	°C.
June 8	10.35	Injected 1.5 mg. of morphine sulfate per kilo subcutaneously.						
	11.19	Cannulas have been introduced into the left carotid artery and right jugular vein under ethyl chloride.						
	11.24							37.8
	11.43	Catheterized.			116	5.96		
	11.52		0.34	100				
	11.56–12.39	Injected intravenously 114 cc. of dilute emulsion of normal dog's submaxillary gland.						
	p.m.							
	12.41		0.32	95				
	1.08							38.3
	2.14		0.33	94				
	2.38				100	3.32	1.14	

infusion of pancreas emulsion in this case caused no unfavorable symptoms and had a very striking effect on the glycemia. Before injection there was a constant and moderate hyperglycemia (0.20 and 0.21 per cent). At the end of the injection, the blood sugar had fallen to 0.12 per cent, practically a normal figure, and 1 hour and 39 minutes later the distinctly normal value, 0.08 per cent, was

found. On the next morning hyperglycemia had again been established. The urinary sugar record is also of interest. The excretion of 1.15 gm. of glucose per hour before the injection was followed by a diminution to half that figure during the injection and thereafter only traces were excreted for at least an hour and a half (0.04 gm. per hour). The over night urine also contained very little sugar (0.2 gm. per hour) but it is evident that the kidney was not rendered permanently impermeable to sugar because the next urine obtained by catheter contained 3.62 per cent dextrose.

On the following day (see DP1b in Table V) an injection of another pancreas preparation lowered the blood sugar from 0.27 to 0.20 per cent. The urinary sugar excretion was diminished very markedly; in one period no qualitative reaction whatever could be obtained.

Experiment LP81a.—In Table IV is given an outline of Experiment LP81a, one of the control experiments. The animal received an intravenous injection of submaxillary gland emulsion. The slight decrease in the blood sugar percentage is in marked contrast with the results obtained when pancreas emulsion was used, and runs parallel with the hemoglobin; *i.e.*, here what little effect there is may be referred to dilution by the intravenous injection of 114 cc. of fluid. The urinary data are insufficient in this experiment but indicate a possibly diminished output of sugar.

There were no noticeable general effects caused by the infusion and *on the next day the administration of pancreatic emulsion lowered the blood sugar from 0.38 to 0.24 per cent* (see LP81b, Table V).

In Table V all the experiments with pancreas emulsions are collected. It will be seen that in most of them there occurred a substantial reduction in the blood sugar. As the samples were not taken at short intervals it is quite probable that the lowest values were not discovered. However, normal or nearly normal figures were found in several cases. Study of the table indicates that, when effective, the emulsion produces an effect during the slow injection, because the blood sugar is usually lower at the end of the injection than at the beginning. The glycemia remains low or continues to fall for about $1\frac{1}{2}$ to $1\frac{3}{4}$ hours, and then, if we may trust the few figures at hand, it gradually rises. It is certain that the effect does not last many hours, for in a number of the experiments a sample of blood

TABLE V.

Injection of Pancreatic Emulsions into Depancreatized Dogs.

Experiment No.	Days after operation.	Blood sugar.				Sugar output in urine per hour.					Emulsion prepared from pancreas of same or different animal.	Remarks.
		Before injection.	End of injection.	After injection.		Before injection.	During injection.	After injection.				
		per cent	*per cent*	*min.*	*per cent*	*gm.*	*gm.*	*hrs.*	*gm.*	*cc.*		
LP70	2	0.28	0.09	92	0.08		0.92	1.5	Tr.	1.4	Different.	This dog used for other experiments on preceding day and again 5 days later.
LP72a	1	0.31	0.14	91	0.13		1.85	1.5	0.46	42.5	Same.	Slight symptoms produced by injection (see protocol, Table I). Used 2 days later for Experiment LP72b.
LP72b	3	0.28	0.22	93	0.22		0.88	1.4	0.61	15	"	No symptoms.
LP73	2	0.33	0.25	71	0.16		→	0.8	+	5*	"	Dog died 50 min. after end of injection; heart blood obtained 21 min. later. Peritonitis; perhaps 25 per cent of pancreas not removed.
LP74a	1	0.27	0.21	93	0.18		5.07	1.6	0.66	9	"	Used 4 days later for Experiment LP74b.
LP74b	5	0.23	0.18	92	0.13		1.54	1.5	0.07	5.5	Different.	Dog used on the next day for another experiment.
LP75	2	0.27	0.22	101	0.23		1.14	1.8	1.55	34	"	Killed 3 days later; about 8 per cent of pancreas not removed.
LP76	1	0.33	0.25	97	0.23	3.82	3.20	1.6	0.32	14	Same.	Used this dog 2 days later for another experiment.
LP77a	1	0.21	0.17	95	0.16	1.26	0.66	1.6	0.05	23	"	Ringer's solution used in preparing emulsion; pregnant animal; used 6 days later for LP77c.
LP77c	7	0.24	0.25	90	0.22		→	1.5	0.2*	23.5*	Different.	Dog used 6 days before for LP77a, and 4 days before for LP77b (Table VI).

LP81b	2	0.38	0.26	90	0.24		1.79	1.7	0.09	22.5	Different.	Received injection of submaxillary emulsion on preceding day (Experiment LP81a, Table VI); killed 2 days later; perhaps 6 per cent of pancreas not removed.
DP1a†	1	0.20	0.12	104	0.08	1.16	0.54	1.7	0.04	14	Same.	"Allen" procedure; 8 per cent of pancreas left; used 2 days later for Experiment DP1b.
		0.21						20	0.17	820		
DP1b†	3	0.25	0.21	94	0.20	>1.15	1.25	2.2	0.00	60	Different.	Killed on following day.
		0.27		240	0.21			1.9	0.08	65		
								18	0.44	260		
DP2a†	2	0.31	0.24	107	0.19	1.00	0.88	1.7	0.04	38	Same.	"Allen" procedure; 4 per cent of pancreas left; used 3 days later for Experiment DP2b.
		0.30						18	0.00	425		
DP2b†	5	0.30	0.26	81	0.17	1.20	1.34	1.1	0.00	26	Different.	Had been used 3 days before for Experiment DP2a; used 3 days later for Experiment DP2c.
		0.33‡		164	0.24			1.4	0.42	83		
								18.5	0.55	583		
DP2c†	8	0.33	0.29	140	0.26	1.85	0.26	2.0	0.00	39	Same.	Had been used for Experiments DP2, a and b.
				246	0.30	(24 hrs.)		1.9	0.25	66		

* During and after injection.

† No morphine given.

‡ After feeding.

TABLE VI.

Injections of Emulsions of Tissues, Other than Pancreatic, into Depancreatized Dogs.

Experiment No.	Days after operation.	Blood sugar.				Sugar output in urine per hour.					Tissue used for emulsion.	Remarks.
		Before injection.	End of injection.	After injection.		Before injection.	During injection.	After injection.				
		per cent	*per cent*	*min.*	*per cent*	*gm.*	*gm.*	*hrs.*	*gm.*	*cc.*		
LP77b	3	0.23	0.26	92	0.28	1.07	→	1.8	0.15*	23.5*	Submaxillary gland.	Had been used 2 days before for Experiment LP77a; used 4 days later for Experiment LP77c (see Table V); pregnant.
LP81a	1	0.34	0.32	95	0.33		→	2.0	1.14*	100*	" "	Used on the following day for Experiment LP81b (Table V); perhaps 6 per cent of pancreas not removed.
LP84	1	0.31	0.31	92	0.36		→	1.8	0.08*	2.2*	" "	Required 4.2 mg. of morphine per kilo; considerable depression; died during night; cause of death could not be found at autopsy; 4 per cent of pancreas not removed.
LP86	1	0.39	0.37	92	0.42	1.9	→	1.8	0.27*	11*	" "	Killed on following day.
LP83	1	0.41	0.36	101	0.35		4.75	1.7	2.12	28	Spleen.	Died 3 days later of pulmonary edema.
DP3†	5	0.31	0.32	114	0.32		0.28	3.0	0.20	26	Voluntary muscle.	"Allen" type; at time of this experiment glycosuria was not marked; had been used 4 days before for another experiment; chloroformed 6 weeks later; no morphine used.
				193	0.32			21.5	> 1.9	830		

* During and after injection.

† No morphine given.

was taken on the following morning and invariably showed a hyperglycemic value.

In all except one experiment (LP75), there occurred a diminution in the excretion of sugar. In some cases this was quite marked, resulting in a urine reacting negatively to Benedict's qualitative test. Such urines, however, had normal color and specific gravity. In about half the experiments there was a decided reduction in the vol-

TABLE VII.

Summary.

Maximum Reduction of Blood Sugar Produced by Injection of Tissue Emulsion.

Marked reduction.			Moderate or no reduction.		
Experiment No.	Maximum reduction of blood sugar.	Kind of emulsion.	Experiment No.	Maximum reduction of blood sugar.	Kind of emulsion.
LP70	0.20	Pancreas.	LP72b	0.06	Pancreas.
LP72a	0.18	"	LP75	0.05	"
LP73	0.17	"	LP77a	0.05	"
LP74a	0.09	"	LP77c	0.02	"
LP74b	0.10	"	DP1b	0.07	"
LP76	0.10	"	DP2c	0.07	"
LP81b	0.14	"	LP77b	Increase.	Submaxillary gland.
DP1a	0.13	"	LP81a	0.02	" "
DP2a	0.11	"	LP84	Increase.	" "
DP2b	0.16	"	LP86	0.02	" "
			LP83	0.06	Spleen.
			DP3	Increase.	Muscle.

ume of urine secreted in the $1\frac{1}{2}$ hour period following the injection; indicating perhaps some temporary functional damage to the kidney; it is possible that this may be caused by the injection of any tissue extract. In the rest of the experiments there was no diminution in urinary flow even when the sugar output had practically ceased (see Experiments LP77a, DP1a and b, DP2a, b, and c, Table V).

The controls in which emulsions of several other tissues were injected are tabulated in Table VI. There was no marked effect on the blood sugar in any of these tests. Injection of emulsion of

spleen (Experiment LP83) was followed by a slight fall of blood sugar, from 0.41 to 0.35 per cent; this was the maximum effect observed in any of the controls. Indeed a slight increase in the blood sugar was observed in three experiments. The amount of sugar eliminated by the kidney was usually decreased to some extent, but not so much as in the pancreas injection experiments. In none of these controls, for instance, was a negative or weak Benedict's test obtained and it is probable that in some of these, as in many of the pancreas emulsion experiments, we have to do with an impairment of the renal epithelium. The striking point in these six experiments is the regular failure to observe a marked lowering of the blood sugar as contrasted with the success when pancreas emulsion was used.

This difference is brought out in the summary (Table VII). Here it will be observed that ten out of sixteen pancreas emulsion injections resulted in a marked reduction of the blood sugar, the greatest reduction in each case falling in the range 0.09 to 0.20 per cent. The other six pancreas experiments, as well as all the controls, showed a reduction of only 0.07 per cent or less. Only one of all the sixteen pancreas experiments had practically a negative result (LP77c), while only one of the controls showed anything resembling a positive effect (LP83).

DISCUSSION.

Many investigators[13] have recognized that the best evidence for the internal secretion theory of the origin of diabetes would be an antidiabetic effect of a pancreatic preparation, administered parenterally. The experiments just described show that such a result has been obtained, since the reduction of hyperglycemia is surely an antidiabetic effect, and the reduction of glycosuria is possibly partly of the same nature. The demonstration of the actual combustion of sugar has not been attempted although the additional proof, while not absolutely essential, would be welcome. A favorable effect upon the glycemia and glycosuria has, however, been established and there remain only a few points to be considered.

[13] See, for example, Hédon, E., Travaux de Physiologie, Paris, 1898, 133. Pflüger, E., *Arch. ges. Physiol.*, 1907, cxviii, 271.

The reduction in glycemia is real, that is, it is not due to dilution. The hemoglobin values, given in the protocols, indicate that this is the case; the same holds true for all the pancreas injection experiments, although this has not been recorded in Table V. Furthermore, that it is not an accident, a mere coincidence, is proven by the fact that it occurred to a marked degree in ten out of sixteen experiments, and to some extent in fifteen out of sixteen. The controls include only one with any effect—and that an insignificant one—on blood sugar. In some cases the pancreas emulsion injection resulted in changing a pronounced hyperglycemia to a normal blood sugar. It is quite likely that more of these cases occurred than were discovered, because the blood samples were not taken at short intervals.

No increased glycosuria occurred which could be said to account for the decrease in blood sugar. On the contrary, the elimination of sugar was decreased in every experiment except one, this being one in which the effect on the blood sugar was slight (Experiment LP75, Table V). The effect upon the urinary sugar generally lasted longer than that upon the blood sugar, but specimens of urine obtained by catheterization on the day following the injection usually contained high percentages of sugar. This would indicate that if the injection of an organ extract decreases the permeability of the kidney to sugar by a toxic action, the effect is only temporary. Since in the controls a similar, but less marked, transitory reduction of the urinary sugar output was observed, it is evident that *the mere reduction of glycosuria is no proof of a beneficial effect of any agent.* Observations of other investigators may be cited in this connection. Murlin and Kramer[5] in one experiment found that an intravenous injection of a mixed boiled extract of pancreas and duodenal mucosa produced a marked fall in the sugar output of a diabetic dog, but that there was a *rise* in the blood sugar. Hédon[14] has shown that transfusion of normal blood into diabetic animals may result in a diminished output of sugar with little if any reduction of the blood sugar level. He assumes that the diabetic complex consists of two phases, of which the renal is the first to be affected by the internal

[14] Hédon, E., *Compt. rend. Soc. biol.*, 1911, lxxi, 124.

secretion of the pancreas. Is it not possible, however, that the introduction of a foreign blood had a toxic effect on the kidney? In fact Hédon himself admits that cross circulation between two diabetic dogs also diminishes glycosuria. The same criticism applies to all blood transfusion and related investigations[15,16] in which only the urine was examined. The same question might be asked regarding Scott's work,[4] for he, too, used only the urinary output as an index of the action of his pancreatic extract.

It must be emphasized that the beneficial effects of the pancreas emulsion are not to be referred to sodium carbonate or any other alkali. *No alkali was used in preparing the pancreas emulsions.* The extractions were made with water and the dilutions of the extract, or emulsion, with 0.9 per cent NaCl. The resulting injection fluid was faintly acid to litmus. Our experiments, therefore, cannot be compared with Murlin and Kramer's[5,7] because they always used an alkaline medium. The alkalinity itself is, according to these authors, largely responsible for the beneficial action of the various tissue extracts which they administered. In our experiments, on the other hand, the favorable effect of the pancreatic emulsion must be due to some ingredient peculiar to it, because no alkali was used in its preparation, and because the other tissue emulsions, prepared in a similar manner, had no such favorable effects.

The mechanism of the effect on the blood sugar may be considered briefly. There are several possibilities, corresponding to the several hypotheses for the mechanism of diabetes. The author does not wish to enter here into an extended discussion of these hypotheses, but wishes to point out how the results of this investigation apply in each case. For the "overproduction" theory the injection fluid may be held to contain a substance which decreases the output of sugar by the liver. If one believes that, in diabetes, the cells themselves lack the power to burn sugar—as many investigators do—it

[15] Forschbach, J., *Arch. exp. Path. u. Pharmacol.*, 1908–09, lx, 131. This author united animals of the same litter (parabiosis) and later removed the pancreas of one, without causing more than a mild glycosuria. In one experiment (No. 5, p. 145) he reports a blood sugar of 0.32 per cent while the urine was sugar-free.

[16] Drennan, F. M., *Am. J. Physiol.*, 1911, xxviii, 396.

will be said that the pancreas emulsion contains the substance which stimulates the cells to this activity, or the "amboceptor" (Allen) which enables the cells to anchor sugar to their protoplasm.

Kleiner and Meltzer[17] have indicated another explanation for some of the phenomena of diabetes; *i.e.*, a decreased permeability to sugar of the capillary endothelia and perhaps of other cells as well. Palmer[18] has corroborated this by showing that the striated muscle of diabetic animals has a lower concentration of glucose than that of normal animals, if the respective levels of blood sugar are taken into account. We have also reported that pancreas emulsions enable diabetic animals to handle, in a nearly normal manner, large amounts of intravenously administered dextrose. This was taken to mean that the pancreas emulsion had restored the permeability, thus permitting the sugar to be absorbed and taken care of in the normal manner. The same explanation may be applied to the present experiments. In other words the pancreas emulsion perhaps contains some substance which alters the permeability of the capillary walls, allowing the diabetic sugar to go through them into the tissues. Another factor which may come into consideration is the state of the blood sugar. The author has presented evidence[19] that the diabetic blood sugar is in a combined or poorly diffusible state. This indicates a further difficulty presented to the diabetic organism in its struggle to obtain sugar for its starving tissues. The enzymes present in the pancreas emulsion may be able to break up this combination setting free crystalloid glucose. This can diffuse readily through the tissues which at the same time have been rendered more permeable to it. This last possibility may be tested directly by the method already described.[19]

The question of the general toxicity of the extracts used requires a few words. It might be expected that an unfiltered tissue extract of this sort would produce grave symptoms when injected intravenously. This was not the case, however. The explanation seems to be that the extract was highly diluted and very slowly injected. In but few instances among the entire list of experiments was any

[17] Kleiner, I. S., and Meltzer, S. J., *Proc. Soc. Exp. Biol. and Med.*, 1914–15, xii, 58.

[18] Palmer, W. W., *J. Biol. Chem.*, 1917, xxx, 79.

[19] Kleiner, I. S., *J. Biol. Chem.*, 1918, xxxiv, 471.

depression or other unfavorable symptom observed. Furthermore, most of the animals received several infusions on different days and survived them. One dog died shortly after a pancreas emulsion injection, but death was apparently due to peritonitis. One control died a few hours after the injection of submaxillary emulsion, and another died of pulmonary edema 3 days after spleen emulsion had been administered.

The fact that these pancreas emulsions lower blood sugar in experimental diabetes without causing marked toxic effects indicates a possible therapeutic application to human beings. It is true that the numerous attempts at pancreas therapy have been almost uniformly unsuccessful. However, the methods heretofore used have not resembled the one used in our experiments. This method is based on certain principles, discussed early in this paper, and is extremely simple. It is possible that the temporary effect which it produced in dogs might be duplicated in man and might be useful in certain emergencies. However, it is doubtful whether attempts along this line should be made until further knowledge has been obtained. It is important to know whether a filtered extract would be effective; and particularly whether an emulsion of the pancreas of another species would have its effect when injected into a diabetic dog. Other questions also arise. Do such injections raise the respiratory quotient? Could other paths of injection and other animals be used equally well? Finally the search for the effective agent or agents, their purification, concentration, and identification are suggested as promising fields for further work.

SUMMARY.

Diabetic dogs were given intravenous injections of unfiltered water extracts of fresh pancreas, diluted with 0.9 per cent NaCl solution. The preparation was administered very slowly and usually resulted in a marked decrease in the blood sugar. There was no compensating increase in urinary sugar, but rather a decrease, which may be partly due to a temporary toxic renal effect.

The result is regarded as further evidence for the internal secretion theory of experimental diabetes.

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THE NOSE-LICKING REFLEX AND ITS INHIBITION.

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The chief and obvious purpose of the licking of the nose in animals is to clean its orifices. To accomplish that end the anterior part of the tongue must be freely movable and long, and the space which separates the nose from the mouth must be comparatively short. In animals in which these conditions are present, in dogs for instance, nose-licking is not merely an incidental phenomenon; it is a steady and apparently indispensable companion of the act of drinking, while the nasal orifices are mostly under the surface of the liquid. Furthermore, dogs get the liquid into their mouth not by the mechanism of suction, like in man, but by giving the anterior part of the tongue a spoonlike formation and throwing the liquid into the posterior oral cavity. Under these circumstances it certainly frequently occurs that some of the liquid is thrown into the nasal openings. These conditions obviously interfere with the respiration, and it is apparently a necessity for the animal, in order to facilitate its breathing, to frequently interrupt the drinking and cleanse the openings of the nose by means of licking.

The licking is accomplished by successive coördinated contractions of various voluntary muscles of the anterior part of the tongue which are under the control of the hypoglossus nerves. The entire procedure of the nose-licking makes the impression of being a conscious, voluntary act. Furthermore, the licking movements do not appear when the animal is under the influence of ether or chloroform anesthesia.

As far as we know, the physiological literature contains no indication that these movements may also be of a reflex nature. At the last meeting of the American Physiological Society we reported briefly on

a mechanical method of anesthesia (this Journal, 1919, xlix, 120). The method consists in a carefully applied indirect concussion of the skull over the parietal bones. This procedure, when properly performed, abolishes voluntary motions and all sensory reactions, without affecting the reflexes. For instance, strong pinching of the skin or strong electric stimulations of it or of exposed sensory nerve trunk (supra-orbital or lingual), leaves the animal perfectly quiet and without any evidence that it feels any pain. The reflexes, however, are very little affected, if any. The respiratory and vasomotor centers remain apparently intact; respiration continues in a normal rhythm, and the blood pressure remains unaffected; also the reflexes of these centers are unimpaired, for instance, stimulation of the central end of the vagus nerve causes inhibition of the respiration, and stimulation of the central end of the sciatic nerve causes an unmistakable rise of blood pressure; the eyelid and corneal reflexes appear to react in a normal fashion.

This signifies that in the indirect concussion of the brain, when properly performed, we possess a method which is capable of completely abolishing voluntary motions and sensations without perceptibly interfering with reflex actions. Employing this method, we tested the nature of the nose-licking act. The results which were obtained were constant; they are twofold and are very instructive.

We shall first mention the fact that *compressing in some way*, for instance, by hemostatic forceps, *the tip end of the nose or of the anterior part of the septum, causes a very characteristic nose-licking*. The appearance of this seemingly normal nose-licking movement in an animal which shows no other reaction to a pain-producing stimulus is a surprising sight. The nose-licking movements in such an animal are evidently of reflex origin, a fact which agrees with the previously mentioned observation that reflexes are not abolished under this method of mechanical anesthesia.

The second noteworthy observation is the fact that the nose-licking reflex occurs only after the cessation of the adequate stimulus. *During the pressure no attempt of nose-licking is made, may the pressure be ever so strong*. It seeems to us that these observations express the facts that *during* the stimulation the nose-licking reflex is *inhibited* and that it may make its appearance only after the discontinuation of the

stimulus. These phenomena were obtainable for many hours, that is, as long as the animal was under observation.

In other words, our experiments brought out the instructive facts *that the phenomenon of nose-licking is or may be a purely reflex act, and that this reflex mechanism consists of two parts: an inhibition of nose-licking during stimulation, and the setting in of the nose-licking movements soon after the discontinuation of the pressure stimulus.*

Obviously the afferent path of this reflex is located in the ophthalmic branch of the trifacial nerve, and the nerve fibers of the hypoglossus which convey the motor impulses to the anterior part of the tongue contain the efferent nerve fibers of this coördinated reflex. No doubt there is room for a further study of many details of this new reflex, and the mechanism may have to be considered later from various angles. There is, however, one point of view which we wish to discuss here.

It seems to us that our observations on the nose-licking reflex—namely, that it appears only after discontinuation of the compression of the tip end of the nose or the septum and that during this compression the reflex is inhibited—can be explained best by the following assumptions. First, the afferent path of this reflex consists of two antagonistic nerve fibers, nerves, the stimulation of which causes an impulse for the excitation of the muscles performing nose-licking; and nerve fibers, the stimulation of which causes an inhibition of the motor part of this reflex. Second, that when both kinds of the reflex fibers are stimulated simultaneously, the response of the reflex inhibitory fibers predominates to such a degree as to completely obscure the presence of the impulse of the reflex excitation. Third, that the response of the reflex inhibitory fibers to the stimulation possesses either a very weak after-effect, or none at all, while the stimulation of the reflex exciting fibers continues its effect after cessation of the stimulation, to such an efficient degree as to bring out the nose-licking reflex in a definite fashion. In other words, we interpret our phenomenon by assuming that when both (antagonistic) nerve fibers are stimulated simultaneously by compression, the effect on the inhibitory fibers predominates during stimulation; but the nose-licking reflex makes its appearance after the discontinuation of the stimulation by virtue of the efficient after-effect of the reflex excitation impulses.

The foregoing several assumptions are sufficiently supported by facts well known in the physiology of the nervous system. For instance, the assumption that a simultaneous stimulation of antagonistic nerves may bring out the effect of the inhibitory impulses during stimulation while the exciting action may outlast the stimulation and appear after its cessation, in consequence of the after-effect of the exciting impulse, is well illustrated by the relations of the vagus and accelerans nerves in their action upon the heart. As is well known, stimulation of the peripheral end of the vagus causes inhibition, while stimulation of the accelerans nerve causes an acceleration and augmentation of the heart beats. Now when both nerves are stimulated simultaneously, and the stimulus is also discontinued simultaneously, the heart stops beating during stimulation, while after discontinuing the stimulus, the heart beats more frequently and strongly than before the stimulation. This is due to the experimentally well-established facts that the inhibitory response of the vagus nerves predominates during stimulation and that after cessation of the stimulus a long after-effect of the accelerating nerves comes to the fore. In other words, we have here a well-established instance of results of simultaneous stimulation of two antagonistic nerves in which the inhibitory impulse responds during stimulation and the response of the exciting nerves appears after cessation of the stimulus due to an after-effect of the latter type of nerves. However, it must be borne in mind that in this instance the mentioned characteristic results take place in the periphery, within the heart, and not, as is the case in the nose-licking reflex, within a center located in the central nervous system.

On the other hand, the assumption that a single mechanical stimulus may cause simultaneously inhibitory and exciting reflexes in the spinal cord is illustrated by the facts discovered by Sherrington, namely, that a mechanical stimulus of a flexor muscle may cause simultaneously an excitation of the flexor and an inhibition of the extensor muscles (and *vice versa*)—Sherrington's "reciprocal innervation." But here again this instance differs from our observation on the nose-licking reflex, in that the stimulation of both antagonistic nerve fibers causes effects only during stimulation and not after its cessation, and that the effects of the stimulation become manifest in different groups of muscles; while in the nose-licking reflex, the effect of simultaneous stimulation

of the antagonistic nerves becomes manifest in one and the same muscle group, and further, at different times, the exciting reflex effect appearing after the cessation of the stimulus while the inhibitory reflex effect is active during stimulation.

Another illustrating fact is to be found in the observations of Kronecker and Meltzer on the propagation of the peristaltic wave within the esophagus. Throughout the entire length of the gullet the inhibitory action runs ahead of the action which causes the successive contractions of that organ. But it must be pointed out that in the deglutition mechanism the mentioned phenomenon becomes manifest in muscle fibers which are not under the control of volition; we have no definite knowledge as to the rôle which inhibition may play in the buccopharyngeal part of the mechanism of deglutition.

We may perhaps also refer here to the mechanism of the "self-regulation" of respiration. According to Herring and Breuer, distention of the lung causes an inhibition of the inspiratory and an excitation of the expiratory muscles, while collapse of the lung causes an inhibition of the expiratory and an excitation of the inspiratory muscles. In this theory it is assumed that the collapse of the lung is a stimulus and a specific one for the expiration; in other words, distention and collapse are different stimuli; each stimulus acting separately and specifically on the two antagonistic parts of the respiratory mechanism. Meltzer suggested (Du Bois-Reymond's Arch. f. Physiol., 1892, 340) that in the mechanism of "self-regulation" there is only one stimulus for both antagonistic parts of the respiratory function; it is the distention of the lung which stimulates simultaneously both sets of antagonistic nerves; but both sets of nerve fibers differ in their response to the same stimulus by two characteristics, namely, the responses of the inspiratory fibers *a*, predominate during the stimulation (distention) and *b*, possess very little or no after-effect; while the impulses in the nerve fibers which control the expiratory mechanism are obscured during the stimulation but possess a long and efficient after-effect which becomes manifest after the discontinuation of the stimulus (distention). In other words, the inspiration is due to the predominating response to the stimulation of the inspiratory nerves during the distention, and the expiration is due to the after-effect of the impulses carried by the expiratory nerves which are dormant during the distention. Accord-

ing to this theory of respiration, the elements of the reflex mechanism for the function of respiration are to a great degree similar to those which we have assumed as underlying the nose-licking reflex. In the mechanisms of both reflexes the mechanical stimulus affects two antagonistic sets of nerve fibers simultaneously, but the effect of stimulation of one set of fibers predominates during stimulation and the stimulation carries no after-effect; while the impulses of the antagonistic nerves are dormant during stimulation, but become manifest after discontinuation of the stimulation on account of the long and efficient after-effect.

We believe that the element of the after-effect of stimulation is an important factor in the mechanisms of many functions. However, experimental physiology has hitherto completely neglected the study of the rôle which the after-effects of stimulations may actually play in the functions of the animal body.

SUMMARY.

Indirect concussion of the brain may abolish voluntary movements and the sensation of pain, without affecting reflexes. Indirect concussion, when properly carried out, may offer a useful method for bringing out the presence of new reflexes, especially those of the brain.

Nose-licking, at least in dogs, is a reflex act, the motor part of which, however, may, as in many other reflexes, also be performed voluntarily.

In an animal under mechanical anesthesia, induced successfully by indirect concussion of the brain, a nose-licking reflex may readily be brought about by compression of the tip end of the nose or the anterior part of the septum. This reflex is characteristic in that it appears only *after* the discontinuation of compression; it does not appear *during* the compression.

This characteristic behavior is explained by the assumption that the compression stimulates simultaneously two antagonistic sets of nerve fibers, reflex exciting and reflex inhibitory nerves. The influence of the impulse of the reflex inhibitory fibers predominates during the stimulation (compression); the impulses carried by the reflex exciting fibers are dormant during the stimulation but become manifest after discontinuation of the stimulus by virtue of the long and efficient after-effect of this set of nerve fibers.

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AROMATIC ARSENIC COMPOUNDS. I.

A Plan of Procedure for the Synthesis of Arsenicals for Chemotherapeutic Research.

By WALTER A. JACOBS AND MICHAEL HEIDELBERGER.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Received July 2, 1919.

In the following communications and in those which will appear later, we shall describe certain groups of aromatic arsenic compounds which have been the subject of our studies for several years. In collaboration with Drs. Wade H. Brown and Louise Pearce, who have had charge of the biological phases of the work, we have prepared these substances for the treatment of experimental trypanosome and spirochaete infections.

Although the fundamental types of aromatic arsenic chemistry had been amply studied by Michaelis, the methods at his disposal were such as to limit considerably the development of the field opened up by his researches. The proof by Ehrlich and Bertheim[1] that "atoxyl," obtained by the arsenation of aniline,[2] is the sodium salt of *p*-aminophenylarsonic acid at once led to a great expansion in the study of aromatic arsenic compounds. Ehrlich and those associated with him developed the synthetic possibilities very fully and added greatly to our knowledge in this field. The substances described in their published accounts were mainly those containing one or more of the usual substituents such as amino, hydroxyl, carboxyl, halogen, nitro, and the like on the same aromatic nucleus as either trivalent or pentavalent arsenic.

The great practical achievements of this work were the synthesis of diamino-dihydroxy-arsenobenzene (salvarsan base) and the discovery of its great value in the treatment of syphilis and other related

[1] *Ber.*, **40**, 3292 (1907).

[2] Béchamp, *Bull. soc. chim.*, **5**, 518 (1863).

diseases. Later synthetic attempts to improve upon this drug were mainly conducted along similar lines, involving the synthesis of substances analogous to salvarsan, of derivatives of the latter and of various metallic coördination compounds of these substances. In the majority of instances the studies have involved compounds in which the chemical variations have been confined to the aromatic nucleus containing the arsenic and it can be assumed that such material has been the subject of fairly exhaustive study.

It seemed wise, therefore, to look in other directions for opportunities for the further synthetic development of arsenic compounds rather than to attempt an extension of the already thoroughly studied groups of substances. However, in the formulation of any comprehensive and systematic developmental policy, it was necessary to keep in mind the biological aim of the work, and in planning our synthetic procedure we have accordingly given special consideration to certain desiderata which we believed essential in this connection. Since these requirements have influenced the character of the substances to be described in the following articles, we feel that a preliminary discussion of these considerations will be appropriate here.

In the first place, work in chemotherapy, to be logical, must be based on the assumption that there is a relationship between chemical constitution and biological action. The substances to be chosen should therefore be of such a character as to permit interpretation of the biological action as a function of chemical structure, in order that the data obtained may be used in the further prosecution of the work. This can be consistently carried out only when the compounds studied are sufficiently alike in general structure to reduce to a minimum the number of chemical features which must be considered in contrasting the biological properties of compounds in order to arrive at the chemical cause of the observed variations in action. The conditions for such homogeneity of material can best be realized by confining the studies to a group of closely related substances which may be regarded as derivatives of some parent compound. The various chemical modifications in the series of drugs would then be chiefly in the nature of the addition to the molecule of groups or side chains, readjustments in their relative positions or alterations confined to such groups or side chains.

In the selection of the types of substances due consideration should be given to the technical character of the work involved in their preparation. As the efficient prosecution of work in chemotherapy can be accomplished only by the intimate and parallel coöperation of both the biological and the chemical sides, preparative difficulties can not be allowed to consume a disproportionate amount of time and effort. It is desirable, therefore, that the types chosen represent readily accessible groups of substances. These types also should be of such a character as to permit of ready chemical modification by the addition of new groups or side chains which may in turn be altered at will as regards number, character, or position. It is important when a certain chemical modification is suggested as particularly desirable from a study of the biological results, that the modification be reasonably easy to execute. Otherwise, as has only too often occurred, the work will be brought to a standstill because of the difficulty of developing further a chemical lead which has been presented.

The realization of these requirements can be made easier if the substances chosen are prepared by reactions of a simple type. For this purpose as starting material there is chosen a readily obtainable substance which contains on the one hand the therapeutic element or radical, and on the other hand some reactive group such as, for example, the amino group. Each new compound would then be produced by the reaction of this starting material with a second class of substances, each of which possesses the same suitably reactive group as, for instance, aliphatically bound halogen. The production of the final compounds would in each case depend mainly on the accessibility of this second group of substances. It may not be irrelevant to point out that accessibility is not only an important aid to scientific orientation, but also determines the cost of any remedy which might be developed. Finally, for practical reasons, substances which can be dissolved in water are most desirable.

From a purely chemical standpoint, in applying these requirements to the preparation of arsenic compounds for biological study, it was obvious from the first that pentavalent arsenic compounds would offer the best material for initial explorations. The trivalent arsenic derivatives as a class do not meet with the technical requirements as above set forth whereas the more available, more easily handled, and

more stable pentavalent compounds promised to afford wider opportunities for synthetic work. These substances, possessing the arsenic as the salt-forming arsonic acid residue, at once solved the problem of solubility. For biological work, arseno compounds, on the other hand, are restricted to those which possess other salt-forming groups and are therefore far more limited than the pentavalent compounds. We are inclined to regard reduction to the arseno form more as a chemical modification which could ultimately be attempted where the presence of a non-arsenical solubilizing group might make this desirable.

Arsanilic acid, the most accessible of the aromatic arsenic compounds, was naturally chosen as the fundamental substance. This compound contains not only the therapeutic element arsenic, in a salt-forming combination, but also carries a very reactive amino group. As Ehrlich and Bertheim have amply shown, it can be subjected to all the reactions which characterize primary aromatic amino compounds. It therefore afforded the logical and ideal starting material for the synthetic treatment outlined above. By choosing the proper reaction it was possible to make use of this amino group in such a way as to form a side chain which served the function of a connecting link between the benzene nucleus containing the arsenic and another group in which the chemical alterations were made. Moreover, in particular cases in which an optimal effect had been achieved with a substance derived from arsanilic acid, the opportunity always remained for studying the further effect of changes in position or of the addition of new groups in the nucleus containing the arsenic, by making the analogous substance derived from the isomers, homologs, or other substitution products of arsanilic acid.

p-Hydroxy-phenylarsonic acid, another easily available aromatic arsenic compound, offered similar possibilities by virtue of the hydroxyl group, although this is more limited in reactivity than the amino group.

The following types which were developed in the present work will present this plan of procedure in more concrete form:

I. Diazoamino Compounds, $A-N=N-NRR'$, in which A is the arylarsonic acid radical and R and R′ hydrogen, alkyl, aryl, or substituted aryl groups.

As Ehrlich and Bertheim have shown, arsanilic acid may be readily and quantitatively diazotized and the diazo compound coupled with appropriate couplers to form azo dyes. We have likewise found that the diazo compound couples in a normal manner with suitable secondary aliphatic amines and primary and secondary aromatic amines to form diazoamino compounds. The same result could also be obtained in the case of the primary aromatic amines by first diazotizing these and coupling the resulting diazonium salts with arsanilic acid. The compounds obtained from the aromatic amines suited our purpose best.

II. Azo Dyes, A — N = N — R, in which A is the arylarsonic acid radical and R is the aromatic coupler.

III. N-Substituted Amides of N-Phenylglycine-p-arsonic Acid, A — $NHCH_2CONRR'$ in which R and R′ are hydrogen, alkyl, aryl, or substituted aryl groups.

As a primary amino compound, arsanilic acid should react with alkyl halides to form *N*-alkyl derivatives. In the particular direction in which we chose to employ this reaction, its practicability had already been suggested by the preparation of the single substance, phenylglycine-*p*-arsonic acid, from arsanilic acid and chloroacetic acid.[1] We have demonstrated, as was to be expected, that this reaction could be extended to chloroacetyl compounds in general. Our attention was centered mainly upon the development of a series of compounds by the use of the chloroacetyl derivatives of primary and secondary aliphatic or aromatic amines.

IV. β-Substituted Ureides of N-Phenylglycine-p-arsonic Acid, A — $NHCH_2CONHCONHR$.—This type of substance is closely related to the previous one and was in like manner easily prepared from arsanilic acid and the chloroacetyl-uramino compounds, $ClCH_2CONHCONHR$ in which R may be hydrogen, or an alkyl, aryl, or substituted aryl group.

V. Substituted N-Phenylglycyl Derivatives of Arsanilic Acid, A — $NHCOCH_2NHR$.—Type III naturally suggests a parallel series of compounds in which the side chain joining the two benzene nuclei is reversed. These substances were prepared with remarkable readi-

[1] Ger. pat. 204,664.

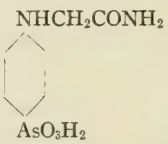
ness from chloroacetylarsanilic acid and primary or secondary amino compounds.

VI. Substituted o-Phenyl Glycollyl Derivatives of Arsanilic Acid.—When the amine in the preceding series was replaced by a phenol in alkaline solution, chloroacetylarsanilic acid was found to react smoothly in the proper sense, yielding substituted *o*-phenyl glycollyl derivatives of arsanilic acid, of the following general formula: $A — NHCOCH_2OR$.

VII. Substituted Amides of o-Phenyl Glycollic Acid p-Arsonic Acid, $A — o\text{-}CH_2CONHR$.—By a reaction identical with that given in Type VI, only starting with *p*-hydroxy-phenylarsonic acid and chloroacetyl-amino compounds, an analogous type of substances was prepared, differing from Type VI in that the side chain connecting the two nuclei is reversed.

Still other types were similarly studied.

Since the development of Type III has led to the most important results in the present work we shall confine the discussion to this group of substances. In this type the fundamental member of the series is *N*-phenylglycineamide-*p*-arsonic acid,



This substance was prepared by the simple reaction of chloroacetamide, on the sodium salt of arsanilic acid. By replacing the chloroacetamide by chloroacetylalkyl amines a limited series of compounds was prepared, which naturally preceded those of the aromatic series. The broadest scope for synthetic work, of course, was furnished by the aromatic series, compounds of this type being obtained from substituted chloroacetylarylamines and arsanilic acid.

This sub-group possesses two aromatic nuclei, an arsenical nucleus and a non-arsenical nucleus. It is apparent that by the choice of the proper chloroacetylarylamine any desired chemical groupings on the non-arsenical nucleus could be obtained. These could be made to vary at will as regards the character, number, and position of the substituting groups. And as the resulting substances were all obtained

by the same reaction the technique of their preparation was easily standardized. The availability of any substance of this series was therefore determined only by the accessibility of the aromatic amine from which the chloroacetyl compound was prepared. As the alterations in the chemical groupings were first confined to the non-arsenical nucleus, each individual substance could be considered to be derived from the same parent type. Insofar as the biological action can be interpreted as a function of constitution, such a series therefore offered the opportunity of determining the biological value of each group, or of the position which it occupied in the nucleus. The correlation of the chemical and biological facts obtained with such homogeneous material offered a good opportunity for concluding in what direction new alterations within the same type should be attempted. And as the type was composed of available substances such dictates were generally possible to follow.

It was thus the purpose at first to confine the chemical alterations to the non-arsenical nucleus. But ultimately by employing a homolog, isomer, or other substitution product of arsanilic acid, modifications on the arsenical nucleus were attempted. Finally the side chain—$NHCH_2CONH$—which serves as the link between the two nuclei was also made the place for chemical alterations without destroying the relationship of the resulting compounds to the original type. This group could be changed to the chain—NHCHRCONH—in which R could be alkyl or aryl. Variations of this nature were generally made, not on a finished substance of Type III, but on an intermediate product; for example, if R is phenyl, such compounds were prepared by reacting arsanilic acid with phenylchloroacetylamino compounds.

By the above treatment of the subject we have attempted to realize the conditions postulated at the start as essential in a logical plan for the synthesis of new arsenicals for biological study. It is our belief that a similar treatment of other leads, where chemically possible, will prove of service in chemotherapeutic investigations.

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AROMATIC ARSENIC COMPOUNDS. II.

The Amides and Alkyl Amides of *N*-Arylglycine Arsonic Acids.

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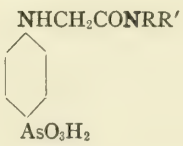
Received July 2, 1919.

As in the case of other aromatic amino compounds, sodium *p*-aminophenylarsonate (sodium arsanilate) has been found to react with chloroacetic acid to form phenylglycine-*p*-arsonic acid.[1] In the present investigations we have found that the amide and alkyl amides of chloroacetic acid react in similar manner to form the amide and alkyl amides of phenylglycine-*p*-arsonic acid, with the following general formula



in which R and R′ may be hydrogen, alkyl, benzyl, or substituted benzyl radicals. Although arsanilic acid itself may be employed in this reaction instead of the sodium salt, the reactivity of the amino group is suppressed by the negative arsonic acid radical so that the reaction proceeds very slowly, and satisfactory yields are only obtained when an extra molecule of the amino acid is employed, since the hydrochloric acid produced during the condensation renders a portion of the base inactive. On the other hand sodium arsanilate exhibits the full reactivity of the amino group and the sodium ion present neutralizes the hydrochloric acid as it is formed.

Chloroacetamide and the simpler chloroacetyl alkylamines condensed readily with sodium arsanilate in boiling aqueous solution, and although the reaction in no instance proceeded quantitatively, owing to the occurrence of side reactions, $\frac{1}{2}$ to 2 hours' boiling sufficed for

[1] Ger. pat. 204,664.

obtaining optimum yields. In the case of the chloroacetylbenzyl amines, 50% alcohol was found to be the most serviceable medium owing to the sparing solubility of these compounds in water. Moreover, the reactivity of the chlorine atom in these substances proved to be so much less than that of the simpler alkyl derivatives that the addition of sodium iodide was found necessary, greatly facilitating the condensation owing to the intermediate formation of the iodoacetyl derivatives.

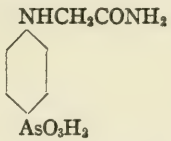
The first member of the series, *N*-phenylglycineamide-*p*-arsonic acid, was also prepared by the action of concentrated ammonia on the methyl ester of phenylglycine-*p*-arsonic acid. This method could unquestionably be employed for the preparation of the simpler homologous compounds, in which case it would necessitate the recovery of the excess of the expensive amines.

The present studies on *p*-arsanilic acid were extended to include analogous substances starting with *o*- and *m*-arsanilic acids, and the homologous aminoaryl arsonic acids.

All of the glycineamide arsonic acids are colorless, crystalline substances which are as a rule but sparingly soluble in the usual neutral solvents and possess high melting or decomposition points, the latter depending greatly upon the rate of heating. As arsonic acids they dissolve in alkalies and alkaline carbonates to form neutral salts, but are completely displaced from these by so weak an acid as acetic acid. Because of the imino group they also possess basic properties and form nitroso derivatives. The basicity appears to be more feeble than that of arsanilic acid itself, since these substances dissolve or form stable hydrochlorides only in strong hydrochloric acid, the salts being easily hydrolyzed by water. On boiling with an excess of alkali or with mineral acids the amide linking is hydrolyzed with the formation of the glycine arsonic acid and the amine.

In the experimental part of this and subsequent papers a description of the sodium salts will be frequently encountered. In many cases these afforded a means for purifying such crude reaction products as could not be directly recrystallized, but the majority were prepared in order to furnish substances in a readily soluble form convenient for biological testing.

Since the results obtained with the first member of the group, *N*-phenylglycineamide-*p*-arsonic acid,



in the treatment of experimental trypanosome and spirochaete infections have been of so promising a character and because of its low toxicity, this substance has been made the subject of extensive study.[1] The biological results are being published elsewhere by our colleagues, Drs. Brown and Pearce. Because of the importance of this compound we are presenting it and the other derivatives obtained from *p*-aminophenyl arsonic acid first, the derivatives of *o*- and *m*-aminophenyl arsonic acids following these.

EXPERIMENTAL.

(*A*) *Derivatives of p-Aminophenyl Arsonic Acid.*

N-(Phenyl-4-arsonic acid)glycineamide (*N-Phenyl-glycineamide-p-arsonic acid*).—Of the two methods used for the preparation of this substance that described first is perhaps to be preferred.

[1] It may be appropriate to mention here that this substance and related compounds, described in the present and following papers of the series, are covered by U. S. Patents, Nos. 1,280,119–27. Patents have also been applied for in foreign countries.

All discoveries made at The Rockefeller Institute are made freely available to the public, in accordance with the philanthropic purposes of the institution. In order to insure purity of product and protection against exploitation, it has been deemed necessary in certain instances to protect the discoveries by patents. It is the purpose of the Institute to permit any drugs which may prove of practical therapeutic value to be manufactured under license by suitable chemical firms and under conditions of production which will insure the biological qualities of the drugs and their marketing at reasonable prices. Other than through the issuance of license, The Rockefeller Institute does not participate in any way in the commercial preparation or sale of the manufactured chemicals; and it receives no royalties or other pecuniary benefits from the licenses it issues.

434 g. of arsanilic acid[1] were dissolved in 2 liters of *N* sodium hydroxide solution. After adding 375 g. of chloroacetamide[2] the mixture was boiled under a reflux condenser for 45 minutes, the clear solution setting to a solid mass of the crude product on cooling. 75 cc. of conc. hydrochloric acid were added to the cold mixture to hold any unchanged arsanilic acid in solution and the substance was then filtered off and carefully washed with cold water. For purification it was suspended in sufficient water to form a thin paste and carefully treated, with stirring, with 25% sodium hydroxide solution until the acid was completely dissolved. The filtered solution was then treated with an excess of acetic acid, whereupon the substance separated as minute, lustrous plates. After filtering, washing thoroughly, and drying, the yield was 300 g. The use of the above amount of chloroacetamide, though twice that theoretically required, was found advisable in order to obtain a good yield. The *acid* is very sparingly soluble in cold water, but dissolves readily on heating. It separates from the hot, aqueous solution in aggregates of long thin plates. It is insoluble in methyl alcohol, acetone, or chloroform, and sparingly in hot methyl or ethyl alcohol, but dissolves in boiling acetic acid. It is sparingly soluble in dil. hydrochloric acid but dissolves readily in the strong acid, its behavior showing it to be a weaker base than arsanilic acid. On boiling its solution in sodium hydroxide ammonia is evolved. When rapidly heated in an open capillary tube it darkens and softens at 280°, but does not melt.

Subs., 0.1405 : 12.35 cc. N (22.0°, 761 mm.). Subs., 0.3205: $Mg_2As_2O_7$, 0.1832.
Calc. for $C_8H_{11}O_4N_2As$: N, 10.22; As, 27.33. Found: N, 10.18; As, 27.59.

Salts.—The pure acid is suspended in enough water to form a thick paste and carefully treated with 25% sodium hydroxide solution until completely dissolved and the solution reacts neutral to litmus. Two

[1] The first portions of this and several other arylarsonic acids were kindly furnished before the war by L. Cassella and Co. and the Farbwerke Höchst. Later, when larger amounts of arsanilic acid were required it was prepared by us by heating aniline and arsenic acid, although we soon adopted a modification of this general method kindly placed at our disposal by Dr. G. W. Raiziss, of the Dermatological Research Laboratories of Philadelphia.

[2] *J. Biol. Chem.*, **21**, 145 (1915).

volumes of alcohol are then added, the pure *sodium salt* quickly separating as thin, nacreous plates. After filtering and washing with 85% alcohol it is air-dried and then contains $\frac{1}{2}$ molecule of water of crystallization. The sodium salt is extremely soluble in cold water, the solution reacting neutral to litmus.

Subs., air-dry, 0.3921: loss, 0.0117 *in vacuo* over H_2SO_4 at 100°.
Calc. for $C_8H_{10}O_4N_2AsNa.0.5H_2O$: H_2O, 2.95. Found: 2.98.
Subs., anhydrous, 0.1503: 12.45 cc. N (25.0°, 762 mm.). Subs., 0.2300: $Mg_2As_2O_7$, 0.1195.
Calc. for $C_8H_{10}O_4N_2AsNa$: N, 9.46; As, 25.32. Found: N, 9.52; As, 25.08.

The *potassium* and *ammonium salts* were prepared in the same way as the sodium salt and form thin, glistening, hexagonal, microscopic platelets. On adding a calcium chloride solution to a solution of the sodium salt the *calcium salt* gradually separates as microscopic, wedge-shaped prisms, containing no water of crystallization. Magnesia mixture causes no precipitate in the cold, but on warming the *magnesium salt* separates as a microcrystalline powder. Heavy metal salts give immediate precipitates, the *silver salt* forming aggregates of thin, microscopic needles.

N-Phenyl-glycineamide-*p*-arsonic acid was also prepared as follows from *N*-phenylglycine methyl ester *p*-arsonic acid by the action of ammonia.

N-(Phenyl-4-arsonic acid)glycine methyl ester, p-$H_2O_3AsC_6H_4NHCH_2COOCH_3$.—40 g. of *N*-phenylglycine-*p*-arsonic acid[1] were treated with 120 g. of dry methyl alcohol and 12 g. of conc. sulfuric acid. The mixture was boiled under a reflux condenser for two hours. The ester separated on cooling and scratching, the precipitation being completed by the addition of water. The filtered, washed, and dried product weighed 38 g. It can be recrystallized from hot water or hot 95% alcohol, separating from the former as microscopic needles and thin plates. It is very sparingly soluble in cold water, cold alcohol, or boiling acetone, and is fairly easily soluble in methyl alcohol, especially on warming. When rapidly heated it softens and darkens above 200° and decomposes at about 285°.

[1] Ger. pat. 204,664.

Subs., 0.1560: 7.0 cc. N (21.0°, 747 mm.). Subs., 0.3135: $Mg_2As_2O_7$, 0.1665. Calc. for $C_9H_{12}O_5NAs$: N, 4.84; As, 25.94. Found: N, 5.12; As, 25.63.

The ester was converted into the amide as follows: 10 g. of the ester were slowly added, with stirring, to 30 cc. of well chilled, conc. ammonia. At first a thick paste of the ammonium salt of the ester was formed, but on allowing the mixture to rise to room temperature the reaction proceeded with formation of a clear solution. After 24 hours the excess of ammonia was removed, preferably *in vacuo*. On diluting with water, filtering, and acidifying with acetic acid, phenylglycineamide-*p*-arsonic acid separated in characteristic form. This was purified as described above and was identical in every way with the product obtained by the direct method. The yield was 80% of the theoretical amount.

Subs., 0.1297: 11.5 cc. N (19.5°, 742 mm.).
Calc. for $C_8H_{11}O_4N_2As$: N, 10.22. Found: 10.11.

N-(Phenyl-4-arsonic acid)glycine ethyl ester, *p*-$H_2O_3AsC_6H_4NHCH_2CO_2C_2H_5$.—Recrystallized from 50% alcohol the ethyl ester forms flat, delicate needles which melt and decompose at about 270° with preliminary darkening and softening. It is very difficultly soluble in cold water, rather more soluble in cold alcohol, but dissolves quite readily in either solvent on boiling.

Subs., 0.1944: (Kjeldahl) 9.3 cc. 0.0714 *N* HCl. Subs., 0.3287: $Mg_2As_2O_7$, 0.1703. Calc. for $C_{10}H_{14}O_5NAs$: N, 4.62; As, 24.72. Found: N, 4.78; As, 25.01.

N-(Phenyl-4-arsonic acid) nitroso-glycineamide, *p*-$H_2O_3AsC_6H_4N(NO)CH_2CONH_2$.—3 g. of the air-dry sodium salt were dissolved in 10 cc. of water and 2 cc. of 5 *N* sodium nitrite solution (1 mol.) added. On addition of 5 cc. of 1:1 hydrochloric acid (a little over 2 mols.) the rapid separation of characteristic crystals of unchanged phenyl-(4-arsonic acid)-glycineamide occurred, but on gentle warming the crystals suddenly changed to delicate needles and the mixture set to a solid cake. After diluting somewhat with water the cooled mixture was filtered, and the nitroso compound recrystallized from water, in which it is readily soluble at the boiling point and only sparingly in the cold. It forms rosets and sheaves of silky needles which intumesce at 182–3°, with preliminary yellowing, when rapidly heated to

180°, then slowly. The acid is difficultly soluble in cold methyl or ethyl alcohol, more easily on boiling, and dissolves only sparingly in cold acetic acid although quite readily in the boiling acid. It turns yellow under sulfuric acid, dissolving to an almost colorless solution giving a brown-red Liebermann test.

Subs., 0.1116: 13.6 cc. N (25.5°, 758 mm.). Subs., 0.2763: $Mg_2As_2O_7$, 0.1412.
Calc. for $C_8H_{10}O_5N_3As$: N, 13.87; As, 24.72. Found: N, 13.90; As, 24.65.

N-(Phenyl-4-arsonic acid)glycine methyl amide, p-$H_2O_3AsC_6H_4$-$NHCH_2CONHCH_3$.—44 g. of arsanilic acid dissolved in 200 cc. of *N* sodium hydroxide solution,[1] and 25 g. of chloroacetyl-methylamine[2] were boiled under a reflux condenser for one hour. The acid separated from the solution on cooling and scratching and was purified by dissolving in just enough dilute sodium hydroxide solution and reprecipitating with acetic acid. The substance separated as a thick mass of thin, microscopic plates. The yield was 31 g. The acid is fairly easily soluble in hot water, from which it separates as aggregates of curved spears. It is difficultly soluble in methyl alcohol and may be recrystallized from hot 50% alcohol. It darkens and softens above 240° and decomposes after a few moments at 285°.

Subs., 0.3269: (Kjeldahl) 22.4 cc. 0.1 *N* HCl; $Mg_2As_2O_7$, 0.1755.
Calc. for $C_9H_{13}O_4N_2As$: N, 9.73; As, 26.00. Found: N, 9.60; As, 25.91.

N-(Phenyl-4-arsonic acid)glycine ethyl amide, p-$H_2O_3AsC_6H_4$-$NHCH_2CONHC_2H_5$.—44 g. of arsanilic acid and 30 g. of chloroacetyl-ethylamine,[3] were condensed as in previous examples. For purification the resulting ethylamide was dissolved in a small volume of hot water, from which it separated on cooling as a thick crust of platelets. The yield was 30 g. The acid is sparingly soluble in cold water or alcohol, but dissolves readily on warming. It is soluble in methyl alcohol, particularly on warming. The substance darkens above 250° and decomposes at 278–80°. Attempts to make a pure sodium salt were unsuccessful owing to its great solubility in water or dilute alcohol.

[1] Hereafter unless otherwise stated arsanilic acid was always dissolved in the equivalent amount of *N* sodium hydroxide solution and likewise equivalent quantities of the halide were employed for the reaction.

[2] This Journal, **41**, 472 (1919).

[3] *J. Biol. Chem.*, **21**, 149 (1915).

Subs., 0.1441: 11.6 cc. N (21.0°, 762 mm.). Subs., 0.3707: $Mg_2As_2O_7$, 0.1923.
Calc. for $C_{10}H_{15}O_4N_2As$: N, 9.27; As, 24.81. Found: N, 9.37; As, 25.04.

N-(Phenyl-4-arsonic acid)glycine n-propyl amide, p-$H_2O_3AsC_6H_4NHCH_2CONHCH_2CH_2CH_3$.—4.4 g. of arsanilic acid and 3 g. of chloroacetyl-propylamine[1] yielded after two hours an oil which crystallized when rubbed. This was purified by dissolving in hot, dil. ammonia and acidifying with acetic acid. It separated slowly on cooling as flat needles or plates and wedge-shaped prisms. The yield was 3.5 g. The substance is sparingly soluble in boiling water, but readily so in boiling 50% alcohol, from which it separates on cooling as sheaves of microscopic needles. It does not melt below 280°.

Subs., 0.1491: 11.6 cc. N (22.5°, 758 mm.). Subs., 0.3188: $Mg_2As_2O_7$, 0.1549.
Calc. for $C_{11}H_{17}O_4N_2As$: N, 8.86; As, 23.70. Found: N, 8.96; As, 23.45.

N-(Phenyl-4-arsonic acid)glycine dimethyl amide, p-$H_2O_3AsC_6H_4NHCH_2CON(CH_3)_2$.—After ½ hour's boiling the reaction product from 3 g. of chloroacetyl-dimethylamine[2] suddenly separated as a mass of thin, microscopic needles, often occurring in characteristic sheaves. After cooling the mixture was acidified with hydrochloric acid, filtered, and the substance washed with water. It was recrystallized from 50% alcohol, requiring a large volume and separating in the same characteristic form. The yield was 3 g. The substance is very sparingly soluble in the neutral solvents, and when rapidly heated, decomposes at about 241–2°.

Subs., 0.1392: 11.1 cc. N (22.0°, 762 mm.). Subs., 0.3440: $Mg_2As_2O_7$, 0.1747.
Calc. for $C_{10}H_{15}O_4N_2As$: N, 9.27; As, 24.81. Found: N, 9.25; As, 24.50.

Sodium Salt.—A neutral solution of the acid in dil. sodium hydroxide, on treating with two volumes of alcohol, yielded the salt as elongated plates, which after washing with 50% alcohol and air-drying contained 4 molecules of water of crystallization and dissolved easily in water.

Subs., air-dry, 0.4277: loss, 0.0782 *in vacuo* at 80° over H_2SO_4.
Calc. for $C_{10}H_{14}O_4N_2AsNa.4H_2O$: H_2O, 18.19. Found: 18.28.
Subs., anhydrous, 0.2985: (Kjeldahl) 18.35 cc. 0.1 *N* HCl.
Calc. for $C_{10}H_{14}O_4N_2AsNa$: N, 8.65. Found: 8.61.

[1] This Journal, **41**, 472 (1919).
[2] *J. Biol. Chem.*, **21**, 148 (1915).

N-(Phenyl-4-arsonic acid)glycine diethyl amide, p-$H_2O_3AsC_6H_4NHCH_2CON(C_2H_5)_2$.—After one hour 4 g. of chloroacetyl-diethylamine[1] yielded an oil on cooling which soon crystallized. This was purified by dissolving in just enough dil. sodium hydroxide solution and reprecipitating with acetic acid, forming microscopic aggregates of short needles. The yield was 4 g. When rapidly heated it sinters and darkens above 195° and melts at 199–201° with gas evolution. It is difficultly soluble in boiling water but dissolves in boiling methyl alcohol or 50% alcohol.

Subs., 0.3184: (Kjeldahl) 18.95 cc. 0.1 *N* HCl; $Mg_2As_2O_7$, 0.1497.
Calc. for $C_{12}H_{19}O_4N_2As$: N, 8.49; As, 22.69. Found: N, 8.34; As, 22.68.

N-(Phenyl-4-arsonic acid)glycine piperidide, p-$H_2O_3AsC_6H_4NHCH_2CONC_5H_{10}$.—From 4 g. of chloroacetyl-piperidine[2] there resulted after two hours' boiling a viscous oil which readily crystallized. This was filtered off, washed with water, and finally with 50% alcohol to remove traces of an adhering oil. The residue was then recrystallized from hot 50% alcohol, separating in characteristic sheaves of thin microscopic needles which soften and darken above 200° and decompose at 218–21°. The yield was 3 g. The acid is soluble in hot methyl alcohol and hot 50% alcohol, but very sparingly soluble in hot water.

Subs., 0.1433: 9.95 cc. N (22.5°, 762 mm.). Subs., 0.3055: $Mg_2As_2O_7$, 0.1400.
Calc. for $C_{13}H_{19}O_4N_2As$: N, 8.19; As, 21.90. Found: N, 8.05; As, 22.12.

N-(Phenyl-4-arsonic acid)glycine benzyl amide, p-$H_2O_3AsC_6H_4NHCH_2CONHCH_2C_6H_5$.—Since chloroacetyl-benzylamine[3] and its derivatives reacted relatively slowly and incompletely with arsanilic acid, it was found that the reaction could be greatly facilitated by the addition of equivalent amounts of sodium iodide to the reaction mixture, owing to the intermediate formation of the more reactive iodoacetyl derivatives, the medium used being 50% alcohol.

4.4 g. of arsanilic acid dissolved in 20 cc. of *N* sodium hydroxide solution, 3.7 g. of chloroacetyl-benzylamine, 4 g. of sodium iodide, and 20 cc. of alcohol were heated under a reflux condenser for 4 hours.

[1] *J. Biol. Chem.*, **21**, 149 (1915).
[2] This Journal, **41**, 473 (1919).
[3] *J. Biol. Chem.*, **20**, 686 (1915).

On cooling the clear solution thickened to a paste of crystals. The collected reaction product was purified by dissolving in sufficient dil. sodium hydroxide solution and acidifying with acetic acid and again recrystallized from hot 85% alcohol, from which it slowly separated as microscopic needles. The yield was 3 g. The acid is soluble in boiling 50% or 85% alcohol and in boiling methyl alcohol, but very sparingly in boiling water. It decomposes at 282–4°.

Subs., 0.2952: (Kjeldahl) 16.25 cc. 0.1 *N* HCl; $Mg_2As_2O_7$, 0.1250.
Calc. for $C_{15}H_{17}O_4N_2As$: N, 7.70; As, 20.58. Found: N, 7.71; As, 20.43.

N-(Phenyl-4-arsonic acid)glycine 3'-carboxamido benzyl amide, p-$H_2O_3AsC_6H_4NHCH_2CONHCH_2C_6H_4CONH_2$*(m-)*.—In a similar manner 22.5 g. of *m*-carboxamido-chloroacetyl-benzylamine[1] gave a clear solution which on standing and scratching deposited 27.5 g. of crude product. This was purified by dissolving in the requisite amount of dil. sodium hydroxide solution or ammonia and reacidifying with acetic acid. It separated gradually as thick aggregates of microscopic needles. On continued washing with water it tends to become colloidal. The arsonic acid decomposes at 237–9° with preliminary darkening. It is sparingly soluble in hot water and boiling acetic acid and practically insoluble in boiling alcohol.

Subs., 0.3431: (Kjeldahl) 25.42 cc. 0.1 *N* HCl. Subs., 0.3693: $Mg_2As_2O_7$, 0.1400.
Calc. for $C_{16}H_{18}O_5N_3As$: N, 10.32; As, 18.40. Found: N, 10.37; As, 18.29.

Sodium Salt.—The acid was suspended in a small volume of hot water and 25% sodium hydroxide solution added cautiously until the solution cleared and reacted neutral to litmus. On chilling the sodium salt separated in rosets and sheaves of delicate needles, which were filtered off, washed with ice water, and air-dried. The salt separates with 5 molecules of water of crystallization and is freely soluble in water and salted out from concentrated solutions by sodium acetate.

Subs., air-dry, 0.8918: loss, 0.1520 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{16}H_{17}O_5N_3AsNa.5H_2O$: H_2O, 17.35. Found: 17.05.
Subs., anhydrous, 0.3929: (Kjeldahl) 27.35 cc. 0.1 *N* HCl.
Calc. for $C_{16}H_{17}O_5N_3AsNa$: N, 9.79. Found: 9.75.

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[1] *J. Biol. Chem.*, **20**, 694 (1915).

N-(Phenyl-4-arsonic acid)glycine 4'-acetamino benzyl amide, *p*-$H_2O_3AsC_6H_4NHCH_2CONHCH_2C_6H_4NHCOCH_3$(*p*-).—After several hours' boiling the arsonic acid resulting from 4.8 g. of *p*-acetaminochloroacetyl-benzylamine,[1] separated from the hot solution, and after washing with 50% alcohol amounted to 5.5 g. It was purified by reprecipitating its solution in dil. ammonia with acetic acid, separating as flat, microscopic needles. The acid is almost insoluble in the usual solvents except hot 50% alcohol from which it separates on cooling as diamond-shaped plates. It darkens and sinters partly but does not melt below 280°.

Subs., 0.3024: (Kjeldahl) 21.4 cc. 0.1 *N* HCl. Subs., 0.3824: $Mg_2As_2O_7$, 0.1399.
Calc. for $C_{17}H_{20}O_5N_3As$: N, 9.98; As, 17.78. Found: N, 9.91; As, 17.65.

Sodium Salt.—On treatment of the clear, neutral solution of the acid in dil. sodium hydroxide with several volumes of alcohol and scratching, the salt separated as microscopic needles. These were filtered off, washed with 95% alcohol, and air-dried, then containing 4.5 molecules of water of crystallization. The salt is readily soluble in water.

Subs., air-dry, 0.6157: loss, 0.0965 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{17}H_{19}O_5N_3AsNa.4.5H_2O$: H_2O, 15.46. Found: 15.67.
Subs., anhydrous, 0.2974: (Kjeldahl) 20.2 cc. 0.1 *N* HCl; $Mg_2As_2O_7$, 0.1050.
Calc. for $C_{17}H_{19}O_5N_3AsNa$: N, 9.48; As, 16.91. Found: N, 9.52; As, 17.03.

N-(Phenyl-4-arsonic acid)glycine 3'-carboxureido benzyl amide, *p*-$H_2O_3AsC_6H_4NHCH_2CONHCH_2C_6H_4CONHCONH_2$(*m*-).—During the condensation with sodium arsanilate the almost insoluble *m*-(ω-chloroacetylaminomethyl)benzoyl-urea[2] gradually changed its appearance owing to the deposition of the reaction product. After 4 hours the mixture was diluted with water, made alkaline with ammonia, warmed, and filtered. On acidification with acetic acid the filtrate yielded a colorless, crystalline powder. This was filtered off, redissolved in hot dil. ammonia, boiled with bone black, and the hot filtrate treated with acetic acid. The arsonic acid separated as glistening, microscopic aggregates of delicate needles which decompose at

[1] Einhorn and Mauermayer, *Ann.*, **343**, 299 (1905).
[2] This Journal, **39**, 2432 (1917).

239–40° when rapidly heated and are practically insoluble in the usual neutral solvents. The yield was unusually poor.

Subs., 0.1119: 12.4 cc. N (27.0°, 754 mm.). Subs., 0.3052: $Mg_2As_2O_7$, 0.1042.
Calc. for $C_{17}H_{19}O_6N_4As$: N, 12.45; As, 16.65. Found: N, 12.52; As, 16.48.

N-(Phenyl-4-arsonic acid) 4'-uramino benzyl amide, *p*-$H_2O_3AsC_6H_4NHCH_2CONHCH_2C_6H_4NHCONH_2$(*p*-).—As the reaction mixture obtained from *p*-uramino-chloroacetyl-benzylamine[1] remained clear on cooling, most of the alcohol was boiled off and water added. On cooling an oil separated which gradually crystallized. The filtered product was dissolved in dil. sodium hydroxide solution and exactly neutralized with acetic acid. After standing in the refrigerator for 24 hours the solution was filtered from a slight precipitate, but as the sodium salt could not be obtained crystalline by any of the usual methods, the solution was treated with an excess of acetic acid, again causing the separation of the arsonic acid as an oil which gradually crystallized on standing. The purification process was repeated, resulting in considerable loss, the final yield being rather poor. For analysis the substance was dried *in vacuo* at 100°.

Subs., 0.3747: (Kjeldahl) 34.8 cc. 0.1 *N* HCl; $Mg_2As_2O_7$, 0.1360.
Calc. for $C_{16}H_{19}O_5N_4As$: N, 13.27; As, 17.76. Found: N, 13.01; As, 17.52.

N-(Phenyl-4-arsonic acid)glycine 3'-methyl-4'-acetamino benzyl amide, *p*-$H_2O_3AsC_6H_4NHCH_2CONHCH_2C_6H_3(CH_3)NHCOCH_3$ (*m'*, *p'*-).—3-Methyl-4-acetamino-chloroacetyl-benzylamine[2] yielded a reaction mixture which set to a gelatinous mass on cooling. This was broken up, filtered, and washed first with 50% alcohol, then with water. In order to obtain the acid in crystalline form it was found necessary to pass through the *sodium salt*, a process which caused a great reduction in the yield. The amorphous product was first partly purified by solution in dil. sodium hydroxide and reprecipitation with acetic acid. After filtering and washing, the precipitate was dissolved in a small volume of dil. sodium hydroxide solution, carefully neutralized with acetic acid, and treated with a large volume of absolute alcohol. On standing in the refrigerator the sodium salt separated as a vo-

[1] This Journal., **39**, 2442 (1917).
[2] *J. Biol. Chem.*, **20**, 688 (1915).

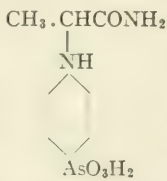
luminous, almost gelatinous mass of delicate, microscopic needles. This was filtered off, recrystallized from 85% alcohol, and air-dried.

The analysis of this salt indicated that the air-dry product contained 6 molecules of water of crystallization, of which only 4.5 molecules were removed at 100° *in vacuo* over sulfuric acid.

Subs., air-dry, 0.7383: loss, 0.1053 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{18}H_{21}O_5N_3AsNa.6H_2O$: 4.5 H_2O, 14.32. Found: 14.26.
Subs., dried, 0.1214: 9.5 cc. N (26.5°, 751 mm.). Subs., 0.3410: $Mg_2As_2O_7$, 0.1109.
Calc. for $C_{18}H_{21}O_5N_3AsNa.1.5H_2O$: N, 8.68; As, 15.48. Found: N, 8.81; As, 15.70.

On treating the solution of the sodium salt with acetic acid the *arsonic acid* deposited slowly and was obtained as aggregates of flat, minute needles on recrystallization from 50% alcohol. When rapidly heated it decomposes at 278° and is soluble in boiling water and boiling 50% alcohol.

Subs., 0.1422: 11.7 cc. N (21.5°, 756 mm.).
Calc. for $C_{18}H_{22}O_5N_3As$: N, 9.66. Found: 9.49.

N-(Phenyl-4-arsonic Acid)-α-amino-propionamide,  .—

22 g. of arsanilic acid dissolved in 100 cc. of *N* sodium hydroxide solution, and 22 g. of α-bromo-propionamide were boiled for ¾ hour. The arsonic acid separated on cooling. 40 cc. of 10% hydrochloric acid were added to hold unchanged arsanilic acid in solution and the crystals filtered off and washed. After reprecipitating the solution of the acid in dil. sodium hydroxide with acetic acid 18 g. were obtained. The amide is appreciably soluble in water at room temperature and readily on boiling, crystallizing on cooling as long, thin, hexagonal plates. It is also soluble in hot 50% alcohol. When rapidly heated it darkens above 255° and decomposes at 262–3.5°.

Subs., 0.1526: 12.75 cc. N (22.5°, 765 mm.). Subs., 0.2981: $Mg_2As_2O_7$, 0.1595.
Calc. for $C_9H_{13}O_4N_2As$: N, 9.73; As, 26.00. Found: N, 9.72; As, 25.81.

Sodium Salt.—A neutral solution of the acid in dil. sodium hydroxide was concentrated to dryness *in vacuo* and the residue dissolved in boiling 95% alcohol. On standing in the refrigerator the salt separated from the filtered solution as long, flat, microscopic needles, which contained approximately 2.5 molecules of water of crystallization after air-drying.

Subs., air-dry, 0.5718: loss, 0.0761 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_9H_{12}O_4N_2AsNa.2.5H_2O$: H_2O, 12.68. Found: 13.31.
Subs., anhydrous, 0.1443: 11.00 cc. N (20.5°, 765 mm.). Subs., 0.3333: $Mg_2As_2O_7$, 0.1643.
Calc. for $C_9H_{12}O_4N_2AsNa$: N, 9.03; As, 24.16. Found: N, 8.93; As, 23.79.

Oxanilamide-p-arsonic acid (*p-oxamylaminophenylarsonic acid*), *p*-$H_2O_3AsC_6H_4NHCOCONH_2$.—This substance, although not a glycine derivative, was suggested by its analogy to phenylglycineamide-*p*-arsonic acid, and is, therefore, described here for lack of a more appropriate place. 5 g. of anhydrous sodium arsanilate and 10 g. of ethyl oxamate were heated for 2 hours at 140–50°. The mixture was digested with water, very little going into solution, then acidified with acetic acid and filtered and washed with water. The crude mass was dissolved in dil. ammonia, and on acidifying the warm filtrate with acetic acid the substance slowly recrystallized as felted masses of minute needles in a yield of 2.5 g. It does not darken or melt when heated to 280° and is very sparingly soluble in boiling water or 50% alcohol. The solution in dil. sodium hydroxide evolves ammonia on boiling.

Subs., 0.1546: 12.8 cc. N (22.5°, 761 mm.). Subs., 0.3022: $Mg_2As_2O_7$, 0.1613.
Calc. for $C_8H_9O_5N_2As$: N, 9.73; As, 26.01. Found: N, 9.58; As, 25.76.

(*B*) *Derivatives of o- and m-Aminophenylarsonic Acids.*

N-(Phenyl-2-arsonic acid)glycineamide.—8.8 g. of *o*-aminophenylarsonic acid (*o*-arsanilic acid)[1] dissolved in 40 cc. of *N* sodium hydroxide solution and 7.5 g. of chloroacetamide yielded after $\frac{3}{4}$ of an hour the glycineamide, which crystallized on cooling. The mixture was acidified to congo red with hydrochloric acid and allowed to sepa-

[1] This Journal, **40**, 1583 (1918).

rate more completely in the refrigerator overnight. After filtration and washing with cold water it was dissolved in a small volume of dil. ammonia and acidified to congo red with hydrochloric acid. On standing in the ice-box the arsonic acid separated as flat, microscopic needles. The yield was 5 g. In contradistinction to the *meta* and *para* isomers the *ortho* acid is not displaced completely from its salts by so weak an acid as acetic acid. The substance is soluble in hot 50% alcohol and boiling water and separates from the latter as sheaves of long, thin, narrow plates. It is sparingly soluble in hot methyl and ethyl alcohols. When rapidly heated it decomposes at 198–9°.

Subs., 0.1405: 12.2 cc. N (23.5°, 756 mm.). Subs., 0.2451: $Mg_2As_2O_7$, 0.1372.
Calc. for $C_8H_{11}O_4N_2As$: N, 10.22; As, 27.33. Found: N, 9.96; As, 27.00.

N-(Phenyl-3-arsonic acid)glycineamide.—A solution of 25 g. of *m*-arsanilic acid[1] in 115 cc. of *N* sodium hydroxide solution, and 21 g. of chloroacetamide were boiled for 45 minutes. On standing in the refrigerator the clear solution gradually deposited the reaction product as a hard crust of aggregates of needles which were contaminated with unchanged chloroacetamide. On stirring and rubbing crystallization rapidly completed itself. The substance was filtered off, washed with ice water and dried. The powdered substance was leached out with dry acetone to remove chloroacetamide and then recrystallized from a small volume of hot water. It separated slowly as aggregates of prismatic needles in a yield of 18.5 g. When rapidly heated to 170° and then slowly the substance melts at 175–7° to a liquid filled with bubbles. It is quite soluble in cold water and very easily so on warming. It is also readily soluble in hot ethyl or methyl alcohol, or glacial acetic acid, but practically insoluble in hot acetone.

Subs., 0.1334: 11.8 cc. N (24.0°, 765 mm.). Subs., 0.3140: $Mg_2As_2O_7$, 0.1783.
Calc. for $C_8H_{11}O_4N_2As$: N, 10.22; As, 27.33. Found: N, 10.23; As, 27.42.

N-(Phenyl-3-arsonic acid)glycine methyl amide.—12 g. of chloroacetyl-methylamine[2] yielded after one hour's boiling a mixture from which the methyl amide separated slowly on cooling and scratching. On recrystallizing from hot water, in which it is readily soluble, it

[1] This Journal, **40**, 1583 (1918).
[2] *Ibid.*, **41**, 472 (1919).

separated as aggregates of flat, microscopic needles or platelets. The yield was 13.8 g. The substance is sparingly soluble in cold water or 50% alcohol, readily on warming. When rapidly heated it darkens and melts at 193–4.5° with gas evolution.

Subs., 0.1452: 12.45 cc. N (22.5°, 753 mm.). Subs., 0.3659: $Mg_2As_2O_7$, 0.1986. Calc. for $C_9H_{13}O_4N_2As$: N, 9.73; As, 26.00. Found: N, 9.82; As, 26.21.

(*C*) *Derivatives of Aminotolylarsonic Acids.*

N-(2-Methylphenyl-5-arsonic acid)glycineamide.—After one hour's boiling the product from 4.6 g. of 3-amino-4-methylphenyl arsonic acid[1] and 3.8 g. of chloroacetamide slowly crystallized on cooling and acidifying with hydrochloric acid. Reprecipitated from ammoniacal solution by acetic acid it separates slowly as delicate, interlaced needles which do not melt below 285°. It is sparingly soluble in water, alcohol, or acetic acid in the cold, but readily on warming. Methyl alcohol dissolves the substance at room temperature.

Subs., 0.1702: (Kjeldahl) 11.65 cc. 0.1 *N* HCl. Subs., 0.2520: $Mg_2As_2O_7$, 0.1347. Calc. for $C_9H_{13}O_4N_2As$: N, 9.73; As, 26.00. Found: N, 9.59; As, 25.79.

N-(2-Methylphenyl-4-arsonic acid)glycineamide.—As in the preceding case 23 g. of 1-amino-2-methylphenyl-4-arsonic acid (from *o*-toluidine) and 19 g. of chloroacetamide yielded the glycineamide. This was purified by solution in dil. ammonia and reprecipitation with acetic acid, separating as aggregates of glistening platelets which darken above 250° and decompose at about 283°. The yield was 10 g. The substance is sparingly soluble in boiling water but more readily so in boiling 50% alcohol.

Subs., 0.1417: 12.00 cc. N (22.5°, 756 mm.). Subs., 0.2619: $Mg_2As_2O_7$, 0.1393. Calc. for $C_9H_{13}O_4N_2As$: N, 9.73; As, 26.00. Found: N, 9.74; As, 25.67.

N-(3-Methylphenyl-4-arsonic acid)glycineamide.—This substance was prepared in similar manner from 1-amino-3-methylphenyl-4-arsonic acid.[2] The amide is soluble in boiling water, from which it separates as lustrous, diamond-shaped platelets. It is also soluble in boiling 50% alcohol and melts at 203–5° with gas evolution.

[1] This Journal, **40**, 1586 (1918).
[2] *Ibid.*, **40**, 1588 (1918).

Subs., 0.1384: 11.8 cc. N (21.0°, 757 mm.). Subs., 0.3120; $Mg_2As_2O_7$, 0.1668.
Calc. for $C_9H_{13}O_4N_2As$: N, 9.73; As, 26.00. Found: N, 9.87; As, 25.81.

N-(2,5-Dimethylphenyl-4-arsonic acid)glycineamide.—2,5-Dimethyl-4-aminophenyl arsonic acid[1] dissolved in *N* sodium hydroxide solution was boiled for 45 minutes with 2 g. of chloroacetamide. On acidifying to congo red with hydrochloric acid and cooling, the arsonic acid crystallized. The crude product was dissolved in dil. ammonia, treated with bone black, and the filtrate acidified with acetic acid, the arsonic acid separating slowly as aggregates of slightly brownish plates and prisms. When rapidly heated it melts and decomposes at 236–7° with preliminary darkening and softening. It is sparingly soluble in cold water, acetic acid, or 50% alcohol, but dissolves more easily in these solvents on boiling.

Subs., 0.1644: (Kjeldahl) 10.9 cc. 0.1 *N* HCl. Subs., 0.3224: $Mg_2As_2O_7$, 0.1644.
Calc. for $C_{10}H_{15}O_4N_2As$: N, 9.27; As, 24.81. Found: N, 9.29; As, 24.61.

[1] This Journal, **40,** 1590 (1918).

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AROMATIC ARSENIC COMPOUNDS. III.

The Ureides and β-Substituted Ureides of *N*-Arylglycine Arsonic Acids.

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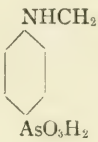
On replacing the amides of chloroacetic acid by the ureide and its β-alkyl or -aryl derivatives in the reaction described in the preceding paper, the ureides and substituted ureides of the arylglycine arsonic acids were obtained,



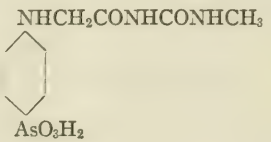
in which R may be hydrogen, an alkyl, or an aryl radical.

When chloroacetyl-urea or its simpler β-alkyl derivatives were employed the reaction could be accomplished by boiling in aqueous solution with the sodium salt of the aminoaryl arsonic acid. In the preparation of the β-arylureides of phenylglycine arsonic acid, however, involving the use of the very sparingly soluble chloroacetyl-substituted phenyl-ureas, the reaction proceeded most satisfactorily in 50% alcohol and only after sodium iodide had been added in order to cause the intermediate formation of the more reactive iodoacetyl compounds.

The new arsonic acids of this series resemble in their general properties the amides of the arylglycine arsonic acids, forming colorless crystalline compounds which are on the whole less soluble than the corresponding substances in the amide series. Like the latter they form stable and soluble neutral salts with the alkali metals. The ureide linkage, like that of the amides, is easily ruptured, this often occurring even at room temperature in solutions containing excess fixed alkali.

Therefore, it was found important to avoid undue exposure to such conditions during the manipulations employed for the preparation of the sodium salts or in the purification of the acids by solution in alkali and reprecipitation with acids. In the latter case the use of dil. ammonia avoided this danger.

Of the numerous substances of this group which were prepared and studied the methylureide of *N*-phenylglycine-*p*-arsonic acid



has been of special interest since it has been found to exert a definite therapeutic effect in experimental syphilis and trypanosomiasis in rabbits.[1] Interesting biological results were also obtained with the ureide itself, although less striking than those given by the methyl derivative. The biological data will be published elsewhere by Drs. Brown and Pearce.

EXPERIMENTAL.

(*A*) *Derivatives of p-Arsanilic Acid.*

N-(Phenyl-4-arsonic acid) glycine-ureide, p-$H_2O_3AsC_6H_4NHCH_2CONHCONH_2$.—44 g. of arsanilic acid, dissolved in 200 cc. of *N* sodium hydroxide solution, and 27 g. of chloroacetyl-urea were boiled under a reflux condenser. After about 15 minutes the reaction product separated. The mixture was heated in a water bath for $\frac{3}{4}$ of an hour longer, cooled, and treated with 50 cc. of conc. hydrochloric acid. The crude, washed ureide was dissolved in dil. ammonia and reprecipitated with acetic acid, separating as aggregates of microscopic needles. It is sparingly soluble in boiling water or 50% alcohol and almost insoluble in methyl alcohol. When rapidly heated it sinters slightly above 230° and darkens, but does not melt below 280°.

Subs., 0.2959: (Kjeldahl) 27.7 cc. 0.1 *N* HCl; $Mg_2As_2O_7$, 0.1445.
Calc. for $C_9H_{12}O_5N_3As$: N, 13.25; As, 23.62. Found: N, 13.12; As, 23.56.

[1] Cf. footnote, THIS JOURNAL, **41**, 1588–9 (1919).

Sodium Salt.—The crude acid obtained above was suspended in a small volume of water and carefully treated with sodium hydroxide until solution was complete and the reaction was neutral to litmus. Addition of too much alkali is to be avoided owing to the danger of hydrolysis. An equal volume of saturated sodium acetate solution was then added, the sodium salt of the arsonic acid separating as well defined hexagonal or diamond-shaped, microscopic platelets. After standing in the refrigerator, the salt was filtered off, washed with a little ice water, and recrystallized from a small volume of hot water. The air-dried salt contains two molecules of water of crystallization and is readily soluble in water. The yield was 30 g. An additional quantity of the arsonic acid was contained in the mother liquors.

Subs., air-dry, 0.3724: loss, 0.0355 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_9H_{11}O_5N_3AsNa.2H_2O$: H_2O, 9.60. Found: 9.53.
Subs., anhydrous, 0.2968: (Kjeldahl) 25.8 cc. 0.1 *N* HCl; $Mg_2As_2O_7$, 0.1372.
Calc. for $C_9H_{11}O_5N_3AsNa$: N, 12.39; As, 22.09. Found: N, 12.18; As, 22.31.

Solutions of the sodium salt give precipitates with salts of the heavy metals. The *silver salt* forms colorless, microscopic needles. Magnesia mixture does not precipitate the solution in the cold but the *magnesium salt* separates on boiling.

N-(Phenyl-4-arsonic acid) glycine-methylureide, *p*-$H_2O_3AsC_6H_4NHCH_2CONHCONHCH_3$.—44 g. of arsanilic acid were dissolved in 200 cc. of *N* sodium hydroxide solution and 30 g. of α-chloroacetyl-β-methyl, urea added. The mixture was boiled for one hour, during which the sparingly soluble chloro compound gradually dissolved. The ureide crystallized on cooling, or in some cases during the heating, after which the mixture was acidified to congo red with hydrochloric acid and filtered. The crude substance was dissolved in dil. ammonia and precipitated by acidifying the solution with acetic acid, but this did not always give satisfactory analytical figures. In order to obtain the acid pure it was converted into the sodium salt as described below and this dissolved in water and again acidified with acetic acid. The substance is sparingly soluble in hot water, separating on cooling as long, thin, glistening needles. It is also difficultly soluble in boiling 50% alcohol and is practically insoluble in the usual organic solvents. When rapidly heated to 220°, then slowly, it decomposes at 224–5°.

Subs., 0.1507: (Kjeldahl) 13.5 cc. 0.1 *N* HCl. Subs., 0.3428: $Mg_2As_2O_7$, 0.1590.
Calc. for $C_{10}H_{14}O_5N_3As$: N, 12.69; As, 22.63. Found: N, 12.54; As, 22.40.

Sodium Salt.—The acid was suspended in a small volume of hot water and carefully treated with sodium hydroxide until the solution reacted neutral to litmus, carefully avoiding an excess of alkali owing to the danger of saponification. After filtering and cooling the sodium salt slowly crystallized and after thorough chilling the salt was filtered off and washed with ice water. It was recrystallized from a small volume of water and air-dried. The yield was 30 g. The sodium salt separates from water as microscopic platelets containing 7 molecules of water of crystallization, although slight impurities, or the conditions under which it is obtained, are apt to affect the water content. It is quite readily soluble in water, exceptionally so on warming, and easily forms supersaturated solutions. The solution of the sodium salt is not precipitated by magnesia mixture in the cold, but yields a precipitate of the *magnesium salt* on boiling. Heavy metal salts cause insoluble precipitates, the *silver salt* separating as thin, microscopic platelets.

Subs., air-dry, 0.7551: loss, 0.2013 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{10}H_{13}O_5N_3AsNa.7H_2O$: H_2O, 26.30. Found: 26.66.
Subs., anhydrous, 0.1438: 15.0 cc. N (24.5°, 754 mm.). Subs., 0.3041: $Mg_2As_2O_7$, 0.1346.
Calc. for $C_{10}H_{13}O_5N_3AsNa$: N, 11.89; As, 21.22. Found: N, 12.04; As, 21.35.

N-(Phenyl-4-arsonic acid) glycine-ethylureide, *p*-$H_2O_3AsC_6H_4NHCH_2CONHCONHC_2H_5$.—Treated as described in the case of the methylureide, 3.3 g. of α-chloroacetyl-β-ethyl-urea[1] yielded 4.5 g. of the ethylureide arsonic acid. The substance is precipitated from the hot solution of its sodium salt (see below) by acetic acid as plumes of microscopic needles which are very difficultly soluble in hot water or methyl alcohol but more readily so in 50% alcohol. When rapidly heated to 220°, then slowly, it decomposes at 223–5°.

Subs., 0.1415: 15.0 cc. N (26.5°, 757 mm.).
Calc. for $C_{11}H_{16}O_5N_3As$: N, 12.17. Found: 12.03.

[1] THIS JOURNAL, **41**, 473 (1919).

Sodium Salt.—An exactly neutral solution of the acid in a small volume of sodium hydroxide solution was treated with several volumes of alcohol. On scratching the salt rapidly separated as a paste of thin, microscopic platelets which were recrystallized from hot 85% alcohol and air-dried. As so obtained the salt contained approximately 4.5 molecules of water of crystallization and dissolved readily in water.

Subs., air-dry, 1.0813: loss, 0.1892 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{11}H_{15}O_5N_3AsNa.4.5H_2O$: H_2O, 18.08. Found: 17.50.
Subs., anhydrous, 0.2950: (Kjeldahl) 23.7 cc. 0.1 *N* HCl; $Mg_2As_2O_7$, 0.1235.
Calc. for $C_{11}H_{15}O_5N_3AsNa$: N, 11.45; As, 20.42. Found: N, 11.26; As, 20.21.

N-(Phenyl-4-arsonic acid) glycine-benzylureide, *p*-$H_2O_3AsC_6H_4NHCH_2CONHCONHCH_2C_6H_5$.—4.4 g. of arsanilic acid were dissolved in 20 cc. of *N* sodium hydroxide solution and 4.6 g. of α-chloroacetyl-β-benzyl urea[1] and 20 cc. of alcohol were added. After 3 hours' boiling, solution was complete and the mixture was chilled, diluted with water, and treated with a slight excess of ammonia. Some insoluble material was filtered off and the filtrate acidified with acetic acid, the benzylureide separating at once. For purification it was again dissolved in dil. ammonia and precipitated by acetic acid. The yield was 2.5 g. Recrystallized from 50% alcohol the acid separated as rosets of delicate needles which, when rapidly heated to 220° and then slowly, decompose at 225°. It is sparingly soluble in boiling water and practically insoluble in methyl alcohol.

Subs., 0.3184: (Kjeldahl) 23.6 cc. 0.1 *N* HCl; $Mg_2As_2O_7$, 0.1205.
Calc. for $C_{16}H_{18}O_5N_3As$: N, 10.32; As, 18.39. Found: N, 10.38; As, 18.26.

α-N-(Phenyl-4-arsonic acid)aminopropionyl-urea, *p*-$H_2O_3AsC_6H_4NHCH(CH_3)CONHCONH_2$.—8.8 g. of arsanilic acid dissolved in 40 cc. of *N* sodium hydroxide solution, and 8 g. of α-bromopropionyl-urea were boiled for one hour. The urea gradually dissolved and the arsonic acid rapidly crystallized on cooling, after which the mixture was treated with 20 cc. of 10% hydrochloric acid and filtered. The resulting substance was reprecipitated from its ammoniacal solution by acetic acid in a yield of 7 g. Recrystallized from 50% alcohol it

[1] *J. Biol. Chem.*, **21**, 152 (1915).

forms aggregates of minute needles which are sparingly soluble in water and methyl alcohol. When rapidly heated to 220°, then slowly, it decomposes at 225–6°.

Subs., 0.1512: 16.7 cc. N (28.0°, 761 mm.). Subs., 0.3142: $Mg_2As_2O_7$, 0.1475.
Calc. for $C_{10}H_{14}O_5N_3As$: N, 12.69; As, 22.63. Found: N, 12.55; As, 22.65.

N-(Phenyl-4-arsonic acid)glycine-phenylureide, *p*-$H_2O_3AsC_6H_4NHCH_2CONHCONHC_6H_5$.—4.4 g. of arsanilic acid were dissolved in 20 cc. of *N* sodium hydroxide solution and 4.4 g. of chloroacetyl-phenylurea, 4 g. of sodium iodide, and 40 cc. of alcohol were added. After 4 hours' boiling the reaction product was filtered off and dissolved in dil. ammonia. The filtered solution was heated and then treated with an excess of acetic acid, causing the separation of the arsonic acid as feathery aggregates of silky hairs. When rapidly heated to 280° the acid darkens slightly and then melts. It is appreciably soluble in boiling 50% alcohol, separating on cooling as long, fine needles, and is almost insoluble in boiling water or methyl alcohol.

Subs., 0.1422: 13.25 cc. N (19.0°, 749 mm.).
Calc. for $C_{15}H_{16}O_5N_3As$: N, 10.69. Found: 10.73.

Sodium Salt.—The acid was suspended in a small amount of hot water and carefully treated with sodium hydroxide solution until just dissolved. The sodium salt separated on cooling as lustrous leaflets. The crystallization was aided by the addition of saturated sodium acetate solution. After filtration and washing with ice water the salt was recrystallized from 50% alcohol, separating on cooling as flat needles containing 5 molecules of water of crystallization.

Subs., air-dry, 0.9424: loss, 0.1701 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{15}H_{15}O_5N_3AsNa.5H_2O$: H_2O, 17.83. Found: 18.05.
Subs., anhydrous, 0.3318: (Kjeldahl) 23.9 cc. 0.1 *N* HCl; $Mg_2As_2O_7$, 0.1251.
Calc. for $C_{15}H_{15}O_5N_3AsNa$: N, 10.13; As, 18.06. Found: N, 10.09; As, 18.20.

N-(Phenyl-4-arsonic acid) glycine-4'-acetamino-phenylureide, *p*-$H_2O_3AsC_6H_4NHCH_2CONHCONHC_6H_4NHCOCH_3$(*p*-).—On boiling 4.4 g. of arsanilic acid dissolved in 20 cc. of *N* sodium hydroxide solution and 20 cc. of alcohol with 5.4 g. of *p*-acetaminophenyl-chloroacetylurea[1] and 4 g. of sodium iodide the sparingly soluble halide was gradu-

[1] THIS JOURNAL, **39**, 1455 (1917).

ally replaced by the reaction product. After 4 hours the solid was almost completely soluble in dil. ammonia, and was then filtered off, dissolved in hot dil. ammonia and precipitated from the hot solution by acetic acid. The arsonic acid separated quickly in clusters of flat, microscopic needles which sinter and darken above 240° when rapidly heated and decompose at 265–6°. The acid is almost insoluble in boiling water but is appreciably soluble in hot 50% alcohol, separating on cooling as plumes of long, delicate hairs.

Subs., 0.1654: (Kjeldahl) 14.65 cc. 0.1 *N* HCl.
Calc. for $C_{17}H_{19}O_6N_4As$: N, 12.44. Found: 12.41.

Sodium Salt.—A concentrated neutral solution of the acid in dil. sodium hydroxide was salted out by the addition of saturated sodium acetate solution and recrystallized from a small volume of boiling water to which a few drops of sodium carbonate solution were added in order to prevent the slight hydrolysis of the salt and separation of the acid, which otherwise occurred in the hot solution. The salt separated on chilling as woolly masses of flat needles which contained 5 molecules of water of crystallization.

Subs., air-dry, 0.6962: loss, 0.1114 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{17}H_{18}O_6N_4AsNa.5H_2O$: H_2O, 16.02. Found: 16.00.
Subs., anhydrous, 0.3165: (Kjeldahl) 26.7 cc. 0.1 *N* HCl; $Mg_2As_2O_7$, 0.1028.
Calc. for $C_{17}H_{18}O_6N_4AsNa$: N, 11.87; As, 15.88. Found: N, 11.82; As, 15.68.

N-(Phenyl-4-arsonic acid) glycine-3'-oxyamylamino-phenylureide, *p*-$H_2O_3AsC_6H_4NHCH_2CONHCONHC_6H_4NHCOCONH_2$ (*m*-).—4.4 g. of arsanilic acid were dissolved in 20 cc. of *N* sodium hydroxide solution and 40 cc. of alcohol and boiled with 4.4 g. of sodium iodide and 6.2 g. of *m*-chloroacetyl-uramino-oxanilamide,[1] the sparingly soluble halide being gradually replaced by the reaction product. After 6 hours the solid was filtered off and digested with dil. ammonia. The filtrate from the undissolved material was diluted to 500 cc., heated on the water bath, and treated with an excess of acetic acid. Precipitated in this manner from the hot solution the arsonic acid separated as a micro-crystalline powder which was easily filtered. The yield was 1.3 g. Although almost insoluble in boiling water the acid

[1] This Journal, 39, 1453 (1917).

dissolves appreciably in hot 50% alcohol. When rapidly heated it decomposes at 223–4°.

Subs., 0.1172: 15.2 cc. N (23.5°, 747 mm.). Subs., 0.2563: $Mg_2As_2O_7$, 0 0819.
Calc. for $C_{17}H_{18}O_7N_5As$: N, 14.62; As, 15.64. Found: N, 14.68; As, 15.42.

N-(Phenyl-4-arsonic acid) glycine-4'-hydroxyphenylureide, *p*-H_2O_3As-$C_6H_4NHCH_2CONHCONHC_6H_4OH$(*p*-).—Because of our inability to prepare satisfactorily *p*-hydroxyphenyl-chloroacetyl-urea the arsonic acid was obtained by using the acetate of this substance. 2.2 g. of arsanilic acid were dissolved in 10 cc. of *N* sodium hydroxide solution and boiled for 2 hours with 2.7 g. of *p*-acetoxyphenyl-chloroacetyl-urea,[1] 2 g. of sodium iodide and 20 cc. of alcohol. On cooling the mixture set to a crystalline cake which was disintegrated, filtered, and washed with 50% alcohol. This product, in which the acetoxy group was presumably still intact, was dissolved in cold, dil. sodium hydroxide solution, making definitely alkaline to phenolphthalein in order to saponify the ester group. The solution was immediately filtered and exactly neutralized with acetic acid, for if allowed to stand too long in alkaline solution the glycylurea grouping is apt to be hydrolyzed as well, giving phenylglycine arsonic acid. From this solution the sodium salt described below was obtained. The acid itself was obtained by dissolving the sodium salts in hot water and treating with an excess of acetic acid, separating at once as microscopic needles containing 1.5 molecules of water of crystallization. The anhydrous substance darkens above 200° and sinters and chars at about 250° when rapidly heated, but does not melt up to 280°.

Subs., air-dry, 0.1851: loss, 0.0113 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{15}H_{16}O_6N_3As.1.5H_2O$: H_2O, 6.20. Found: 6.10.
Subs., anhydrous, 0.1726: (Kjeldahl) 12.80 cc. 0.1 *N* HCl.
Calc. for $C_{15}H_{16}O_6N_3As$: N, 10.27. Found: 10.38.

Sodium Salt.—This was obtained from the solution described above, crystallization being facilitated by the addition of saturated sodium acetate solution. The salt separated as nacreous plates, in a yield of only 1.3 g., owing possibly to the destruction of the substance during the saponification of the acetyl group. When recrystallized from

[1] This Journal, **39**, 2441 (1917).

50% alcohol the salt separates as glistening leaflets containing from 4 to 4.5 molecules of water of crystallization. It is fairly readily soluble in water, particularly on warming.

Subs., air-dry, 0.3698: loss, 0.0560 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{15}H_{15}O_6N_3AsNa.4.5H_2O$: H_2O, 15.81. Found: 15.14.
Subs., anhydrous, 0.2937; (Kjeldahl) 20.5 cc. 0.1 *N* HCl; $Mg_2As_2O_7$, 0.1065.
Calc. for $C_{15}H_{15}O_6N_3AsNa$: N, 9.75; As, 17.39. Found: N, 9.78; As, 17.51.

N-(Phenyl-4-arsonic acid) glycyl-4-uramino-phenoxyacetamide, *p*-$H_2O_3AsC_6H_4NHCH_2CONHCONHC_6H_4OCH_2CONH_2$(*p*-).—In this case, using *p*-chloroacetyluramino-phenoxyacetamide[1] as starting material the reaction mixture behaved as in the preceding examples. After 4 to 5 hours' boiling the mixture was diluted with water, filtered, and the solid suspended in water and treated carefully with dil. sodium hydroxide solution until just alkaline to litmus. The filtrate was exactly neutralized with acetic acid and the *sodium salt* thrown out by the addition of sodium acetate, filtered off, and washed with 20% sodium acetate solution, then with 85% alcohol. Recrystallized from 50% alcohol it separated as aggregates of slightly purplish plates which were practically anhydrous. The salt is fairly readily soluble in water, especially on warming.

Subs., 0.2959: (Kjeldahl) 24.95 cc. 0.1 *N* HCl; $Mg_2As_2O_7$, 0.0929.
Calc. for $C_{17}H_{15}O_7N_4AsNa$: N, 11.48; As, 15.36. Found: N, 11.81; As, 15.16.

On adding acetic acid to a dilute solution of the sodium salt the *arsonic acid* separates slowly as glistening, diamond-shaped platelets which are almost insoluble in boiling water or boiling 50% alcohol. When rapidly heated it decomposes at 243–4° with preliminary sintering and darkening.

Subs., 0.1546: 16.05 cc. N (16.5°, 753 mm.).
Calc. for $C_{17}H_{19}O_7N_4As$: N, 12.02. Found: 12.13.

N-(Phenyl-4-arsonic acid) glycyl-3-uramino-benzamide, *p*-$H_2O_3AsC_6H_4NHCH_2CONHCONHC_6H_4CONH_2$(*m*-).—In the preparation of this substance the sparingly soluble *m*-chloroacetyluramino-benzamide[2] rapidly dissolved as the reaction progressed, and the new arsonic

[1] This Journal, **39**, 2439 (1917).
[2] *Ibid.*, **39**, 2437 (1917).

acid soon separated. After 2 hours' boiling the solid was dissolved in dil. ammonia, the filtrate diluted to several hundred cc., heated, and then acidified with acetic acid. The arsonic acid separated as a rather voluminous, micro-crystalline powder. 4.2 g. were obtained from 5.2 g. of halide. It is very sparingly soluble in boiling water, but somewhat more soluble in boiling 50% alcohol, from which it separates as sheaves of flat, microscopic needles. When rapidly heated it melts at 213–4° with effervescence and darkening.

Subs., 0.1508: 17.0 cc. N (22.5°, 757 mm.). Subs., 0.3042: $Mg_2As_2O_7$, 0.1066.
Calc. for $C_{16}H_{17}O_6N_4As$: N, 12.84; As, 17.18. Found: N, 12.97; As, 16.92.

N-(Phenyl-4-arsonic acid) glycyl-4-uramino-benzamide.—Starting with 5.2 g. of *p*-chloroacetyluramino-benzamide[1] the reaction mixture was boiled for 5 hours. The product obtained was dissolved in dil. ammonia, and the filtrate heated and acidified with acetic acid, the arsonic acid separating as short, microscopic needles. It was further purified by redissolving in dil. ammonia and reprecipitating with acetic acid. The yield was 3.1 g. When rapidly heated the substance darkens above 230° and decomposes at 245°.

Subs., 0.3288: (Kjeldahl) 29.85 cc. 0.1 *N* HCl; $Mg_2As_2O_7$, 0.1160.
Calc. for $C_{16}H_{17}O_6N_4As$: N, 12.84; As, 17.18. Found: N, 12.72; As, 17.03.

N-(Phenyl-4-arsonic acid) glycyl-3-uramino-phenylacetamide, *p*-$H_2O_3AsC_6NHCH_2CONHCONHC_6H_4CH_2CONH_2$(*m*-).—In this case the reaction mixture, containing 10.8 g. of *m*-chloroacetyluramino-phenylacetamide,[1] was boiled for 3 hours. On cooling and scratching the arsonic acid separated from the clear solution as a thick, crystalline mass. After filtering, the crude product was dissolved in dil. ammonia and the filtrate treated with acetic acid, the substance gradually separating as a thick mass of needles in a yield of 9.5 g. The arsonic acid is soluble in hot water from which it separates as felted needles. It is also soluble in 50% alcohol and, when rapidly heated, decomposes at 214–6°.

Subs., 0.1460: 15.9 cc. N (23.0°, 754 mm.). Subs., 0.3102: $Mg_2As_2O_7$, 0.1055.
Calc. for $C_{17}H_{19}O_6N_4As$: N, 12.45; As, 16.65. Found: N, 12.47; As, 16.42.

[1] This Journal, **39**, 2438 (1917).

N-(Phenyl-4-arsonic acid) glycyl-4-uramino-phenylacetamide.—The reaction mixture, containing 8.1 g. of *p*-chloroacetyluramino-phenylacetamide[1] yielded a clear solution after 2 hours' boiling. At the end of 4 hours the solution was cooled and the precipitated solid dissolved by the careful addition of dil. sodium hydroxide solution. After filtering and exactly neutralizing with acetic acid the *sodium salt* was salted out with sodium acetate, filtered off, washed with 85% alcohol, dissolved in a small volume of warm water and treated with an equal volume of alcohol. The sodium salt separated as microscopic, hexagonal platelets containing about 2.5 molecules of water of crystallization. The yield of the air-dry salt was 4.5 g.

Subs., air-dry, 0.3606: loss, 0.0330 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{17}H_{18}O_6N_4AsNa.2.5H_2O$: H_2O, 8.71. Found: 9.15.
Subs., anhydrous, 0.1213: 12.8 cc. N (27.0°, 764 mm.). Subs., 0.2059: $Mg_2As_2O_7$, 0.0667.
Calc. for $C_{17}H_{18}O_6N_4AsNa$: N, 11.87; As, 15.88. Found: N, 12.08; As, 15.64.

On adding acetic acid to the hot solution of the sodium salt the *arsonic acid* separated slowly as plumes of delicate needles containing one molecule of water of crystallization. It is very sparingly soluble in boiling water but more readily so in boiling 50% alcohol. The anhydrous substance decomposes at 218–21° when rapidly heated.

Subs., air-dry, 0.6982: loss, 0.0255 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{17}H_{19}O_6N_4As.H_2O$: H_2O, 3.85. Found: 3.70.
Subs., anhydrous, 0.1485: (Kjeldahl) 13.2 cc. 0.1 *N* HCl.
Calc. for $C_{17}H_{19}O_6N_4As$: N, 12.45. Found: 12.44.

(*B*) *Derivatives of o-Arsanilic Acid.*

N-(Phenyl-2-arsonic acid) glycine-ureide.—4.4 g. of *o*-arsanilic acid[2] were dissolved in 20 cc. of *N* sodium hydroxide solution and boiled ½ hour with 2.7 g. of chloroacetyl-urea, the new arsonic acid precipitating after 7 to 8 minutes. The mixture was cooled, acidified to congo red, filtered, and the solid suspended in a little water, treated with an excess of ammonia, and the solution treated with boneblack and fil-

[1] This Journal, **39**, 2439 (1917).
[2] *Ibid.*, **40**, 1583 (1918).

tered. The pale yellow filtrate, heated to boiling and acidified strongly with acetic acid, deposited the ureide as plumes of minute, delicate needles which are almost insoluble in the usual neutral solvents. The yield was 2.5 g. When rapidly heated to 230°, then slowly, it melts and decomposes at 231–2° with preliminary softening and yellowing.

Subs., 0.1535: (Kjeldahl) 14.4 cc. 0.1 *N* HCl. Subs., 0.3396: $Mg_2As_2O_7$, 0.1647.
Calc. for $C_9H_{12}O_5N_3As$: N, 13.25; As, 23.62. Found: N, 13.14; As, 23.41.

N-(Phenyl-2-arsonic acid) glycine-methylureide.—A solution of 4.4 g. of *o*-arsanilic acid in 20 cc. of *N* aqueous sodium hydroxide solution was boiled 50 minutes with 3.1 g. of α-chloroacetyl-β-methyl-urea, the latter gradually dissolving and the mixture finally setting to an almost solid cake, being then transferred to the water bath and heated $\frac{1}{2}$ hour longer. After reprecipitation from dil. ammonia in the usual manner, 2.1 g. of the arsonic acid were obtained as balls of minute needles which are very sparingly soluble in boiling water, alcohol, or methyl alcohol, but dissolve in boiling acetic acid. When rapidly heated to 215°, then slowly, the acid melts and decomposes at 218° with slight preliminary softening and darkening.

Subs., 0.1642: (Kjeldahl) 15.05 cc. 0.1 *N* HCl. Subs., 0.3248: $Mg_2As_2O_7$, 0.1507.
Calc. for $C_{10}H_{14}O_5N_3As$: N, 12.69; As, 22.63. Found: N, 12.84; As, 22.40.

(*C*) *Derivatives of m-Arsanilic Acid.*

N-(Phenyl-3-arsonic acid) glycine-ureide.—This substance was prepared and purified by the method of the preceding experiment, from 4.4 g. of *m*-arsanilic acid,[1] separating gradually as colorless microscopic needles frequently grouped in bundles. The yield was 2.5 g. It is soluble in boiling water and boiling 50% alcohol and separates from the former in rosets of minute needles. It decomposes at 208–9° with preliminary softening.

Subs., 0.1494: 17.2 cc. N (27.0°, 758 mm.). Subs., 0.3038: $Mg_2As_2O_7$, 0.1501.
Calc. for $C_9H_{12}O_5N_3As$: N, 13.25; As, 23.62. Found: N, 13.08; As, 23.84.

N-(Phenyl-3-arsonic acid) glycine-methylureide.—Using 17.5 g. of *m*-arsanilic acid and 13 g. of α-chloroacetyl-β-methyl-urea in the usual

[1] THIS JOURNAL, **40**, 1583 (1918).

manner, the substance suddenly separated after $\frac{1}{2}$ hour. 80 cc. of *N* hydrochloric acid solution were added on cooling and the precipitate filtered off and washed with water. The yield was 19 g. Recrystallized as in the preceding examples, the acid melts with decomposition at 213–3.5° when rapidly heated. It is rather sparingly soluble in boiling water or 50% alcohol, separating from the former as rosets of delicate needles.

Subs., 0.1460: 16.1 cc. N (23.5°, 750 mm.). Subs., 0.3273: $Mg_2As_2O_7$, 0.1493.
Calc. for $C_{10}H_{14}O_5N_3As$: N, 12.69; As, 22.63. Found: N, 12.54; As, 22.01.

(*D*) *Derivatives of Substituted Arsanilic Acids.*

N-(3-Methylphenyl-4-arsonic acid) glycine-ureide.—16.2 g. of 3-methyl-4-aminophenyl arsonic acid (from *o*-toluidine), were dissolved in 70 cc. of *N* sodium hydroxide solution and boiled with 9.5 g. of chloroacetyl-urea. After $\frac{1}{2}$ hour the mixture suddenly crystallized and was cooled, treated with 25 cc. of 10% hydrochloric acid, filtered, and washed. The acid was dissolved in dil. ammonia, and the solution heated and acidified with acetic acid, causing the separation of the ureide as delicate needles. The yield was 11 g. The substance is sparingly soluble in boiling water or boiling 50% alcohol, and decomposes at about 235° when rapidly heated.

Subs., 0.1392: 15.5 cc. N (22.5°, 756 mm.). Subs., 0.3327: $Mg_2As_2O_7$, 0.1544.
Calc. for $C_{10}H_{14}O_5N_3As$: N,12.69; As, 22.63. Found: N, 12.80; As, 22.40.

Sodium Salt.—The acid was suspended in a small volume of water and sodium hydroxide solution carefully added until solution was complete and the reaction was faintly alkaline to litmus. On warming the solution and adding 2 volumes of alcohol the sodium salt separated as glistening plates containing 2 molecules of water of crystallization. When recrystallized from a small volume of hot water it also separates as the same hydrate.

Subs., air-dry, 0.5114: loss, 0.0460 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{10}H_{13}O_5N_3AsNa.2H_2O$: H_2O, 9.26. Found: 9.00.
Subs., anhydrous, 0.1132: 11.4 cc. N (20.0°, 758 mm.). Subs., 0.2586: $Mg_2As_2O_7$, 0.1123.
Calc. for $C_{10}H_{13}O_5N_3AsNa$: N, 11.90; As, 21.22. Found: N, 11.71; As, 20.96.

N-(3-Methylphenyl-4-arsonic acid) glycine-methylureide.—After one hour 23 g. of 3-methyl-4-aminophenyl arsonic acid as the sodium salt and 15 g. of α-chloroacetyl-β-methyl-urea yielded a solution which set to a thick, crystalline mass on cooling. This was diluted with water and treated with 40 cc. of 10% hydrochloric acid. Reprecipitated from hot dil. ammoniacal solution with acetic acid, the yield was 12 g. The substance is sparingly soluble in hot water but dissolves in boiling 50% alcohol. It separates from the former as hair-like needles and from the latter as radiating masses of minute needles. When rapidly heated it decomposes at 218–9°.

Subs., 0.1326: 14.0 cc. N (24.5°, 756 mm.). Subs., 0.3418: $Mg_2As_2O_7$, 0.1523.
Calc. for $C_{11}H_{16}O_5N_3As$: N, 12.17; As, 21.72. Found: N, 12.07; As, 21.52.

N-(2-Hydroxyphenyl-5-arsonic acid) glycine-ureide.—On boiling a solution of 3.8 g. of 3-amino-4-hydroxyphenyl arsonic acid[1] in 16.5 cc. of *N* sodium hydroxide solution with 4.4 g. of chloroacetyl-urea it suddenly set to a solid, crystalline cake. After another hour on the water bath water was added and the crystalline mass disintegrated and filtered. Reprecipitated from solution in hot, dil. ammonia with acetic acid, the substance separated as flat, minute, almost colorless, glistening needles which contained between 1 and 1.5 molecules of water of crystallization. The yield was 3.3 g. When anhydrous it decomposes at 203–5°.

Subs., air-dry, 0.4214: loss, 0.0257 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_9H_{12}O_6N_3As.H_2O$: H_2O, 5.13. Found: 6.10.
Subs., anhydrous, 0.2946: (Kjeldahl) 26.65 cc. 0.1 *N* HCl; $Mg_2As_2O_7$, 0.1380.
Calc. for $C_9H_{12}O_6N_3As$: N, 12.62; As, 22.50. Found: N, 12.68; As, 22.61.

[1] This Journal, 40, 1590 (1918).

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AROMATIC ARSENIC COMPOUNDS. IV.

Aromatic Amides of *N*-Arylglycine Arsonic Acids.

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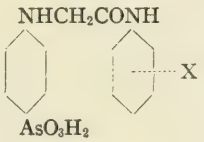
In continuation of the studies described in the preceding papers of this series,[1] we have prepared the arylamides and substituted arylamides of the arylglycine arsonic acids possessing the following general formula:



a series of compounds with such extensive synthetic possibilities as to afford a very fertile ground for chemotherapeutic researches.[2]

Like the alkylamides and ureides these substances were readily obtained by the interaction of the sodium salt of the aminoaryl arsonic acid with the aromatic chloroacetylamino compound, according to the reaction $NaHO_3AsC_6H_4NH_2 + ClCH_2CONHAr \rightarrow H_2O_3AsC_6H_4NHCH_2CONHRAr + NaCl$. In the case of the chloroacetyl derivatives of the simpler amines or of their halogen, nitro, acylamino, carboxamide, carboxureide, or sulfonamide derivatives, the reaction was best accomplished in 50% alcoholic solution in the presence of sodium iodide, the latter causing the intermediate formation of the more reactive iodoacetyl compounds. However, where chloroacetylamino acids or the simpler chloroacetylamino-phenols were employed the condensation could be carried out rapidly in aqueous solution without the use of sodium iodide. In such cases, one-half to one hour's boiling usually sufficed for complete reaction owing to the great lability

[1] This Journal, **41**, 1581, 1587, 1600 (1919).

[2] Cf. footnote, This Journal, **41**, 1588–9 (1919).

of the chlorine in these compounds, whereas the chlorine in the first mentioned substances is far more stable. On boiling the chloroacetyl-amino acids or phenols with aqueous alkali or salts of weak acids a copious liberation of chlorine ion may quickly be obtained, whereas such a result can be obtained with the chloroacetyl compounds of other aromatic amines only through much more vigorous treatment.

In some instances the great reactivity of these chloroacetyl compounds produced complications, since the sodium arsanilate functioned rather as a salt of a weak acid and removed the chlorine before much opportunity for condensation with the amino group was afforded. This difficultly was overcome in the case of chloroacetyl-anthranilic acid by the use of its ethyl ester and subsequent saponification of the resulting ester arsonic acid. It was also found necessary to adopt a similar procedure in the case of chloroacetyl-methylanthranilic acid. A similar difficulty was encountered in using *o*-chloroacetylamino-phenol for the preparation of the *o*-hydroxyanilides, on account of the readiness with which it undergoes *ortho* condensation in the presence of salts of weak acids to form the anhydride of *o*-amino-phenoxy-acetic acid. This reaction preponderated particularly with *o*- and *p*-arsanilic acids and only poor yields of the desired glycine compounds were obtained. The yields were greatly improved, however, by employing two molecules of arsanilic acid itself instead of the sodium salt, the formation of *o*-amino-phenoxyacetic anhydride being almost completely suppressed.

The reaction with *o*-chloroacetylamino-phenol also afforded an interesting opportunity to note the differences in reactivity between *m*-arsanilic acid on the one hand and the *o*- and *p*-acids on the other. It has been our experience that amines with negative substituents such as *m*-nitraniline react more readily with halogen-acetyl compounds than do the *o*- and *p*-isomers. In the reaction between sodium *m*-arsanilate and *o*-chloroacetylamino-phenol the tendency toward glycine formation was such as to proceed at a more rapid rate than the formation of *o*-amino-phenoxyacetic anhydride, so that the yield of the *o*-hydroxyanilide was much greater and that of the by-products much smaller than in the case of the isomeric arsanilic acids. It is probable that a comparative study of the reactivity of the aminophenyl arsonic acids with other halogen-acetyl compounds

would yield a similar result. Such experiments involving velocity measurements were, however, beyond the scope of the present studies.

Special attention has been given in the present work to a study of the hydroxyanilides of the arylglycine arsonic acids, since reduction converts them into a group of arseno compounds which yield water-soluble alkali phenolates. These trivalent arsenicals have been the subject of interesting biological investigations and since both the chemical and biological studies with such compounds are still in progress, their description will be left to later communciations.

As derivatives of the phenylglycine arsonic acids, the substances herein described function both as acids and bases. As acids they are easily displaced from their salts by acetic acid, except in those cases in which the arylamide nucleus is substituted by other acid groups, in which case either a mineral acid or a large excess of acetic acid is required. The feeble basic properties due to the imino group are typified by the formation of hydrochlorides only in the presence of relatively strong hydrochloric acid. As secondary amines they also yield nitroso derivatives. The amide linkage renders the glycine-anilides susceptible to hydrolysis on boiling with mineral acids or alkalies, but they seem somewhat more stable toward the action of alkali than the corresponding aliphatic amides.

As a rule the free arsonic acids do not possess sharp melting or decomposition points, the values obtained depending greatly upon the rate of heating. They are on the whole but sparingly soluble in the usual solvents. The sodium salts, on the other hand, dissolve more or less readily in water depending upon the nature and position of the substituting groups and are often salted out by sodium chloride or acetate. For the convenience of biological testing the sodium salts were prepared in many cases, although this was also found a convenient means for the purification of individual compounds.

EXPERIMENTAL.

(*A*) *Derivatives of p-Arsanilic Acid.*

N-(Phenyl-4-arsonic Acid) glycineanilide, *p*-$H_2O_3AsC_6H_4NHCH_2CONHC_6H_5$.—This substance can be prepared by the two methods already described in the preparation of the amide of phenylglycine-*p*-

arsonic acid;[1] *viz.*, by the reaction of sodium arsanilate with chloroacetanilide or by that of phenyl-(4-arsonic acid)-glycine methyl ester with aniline itself. Both the speed and yield of the first reaction are much improved either by replacing the chloro compound by the iodoacetyl derivative or by adding sodium iodide to the reaction mixture.

22 g. of arsanilic acid were dissolved in 100 cc. of *N* sodium hydroxide solution. To this were added 17 g. of chloroacetanilide,[2] 20 g. of sodium iodide, and 100 cc. of alcohol and the mixture was boiled for two hours. During the heating the reaction product suddenly separated. After cooling the substance was filtered off, washed with 50% alcohol and redissolved in dil. ammonia. On reprecipitating the hot solution with an excess of acetic acid, the arsonic acid separated in easily filterable form in a yield of 25 g. Recrystallized from 50% alcohol it separates as minute, delicate needles which do not melt below 285° when rapidly heated. As a secondary amine it reacts with nitrous acid, giving the nitroso derivative described below. The substance is practically insoluble in the cold in the usual organic solvents, but is appreciably dissolved by a large volume of hot methyl or ethyl alcohol or glacial acetic acid. It is insoluble in cold dil. hydrochloric acid, but on warming it dissolves, forming the hydrochloride, which separates on cooling.

Subs., 0.1308: 9.05 cc. N (22.0°, 763 mm.). Subs., 0.4164: $Mg_2As_2O_7$, 0.1828.
Calc. for $C_{14}H_{15}O_4N_2As$: N, 8.00; As, 21.39. Found: N, 8.04; As, 21.18.

The substance was also prepared as follows: 6 g. of (phenyl-4-arsonic acid)-glycine methyl ester[3] and 12 g. of aniline were treated with 20 cc. of methyl alcohol and heated on the water bath until solution was complete. The alcohol was then allowed to boil off and after several hours' heating the melt began to crystallize. The residue was finally taken up in dil. sodium hydroxide solution, and made definitely alkaline to phenolphthalein to saponify any unchanged ester. The filtered solution was then acidified with acetic acid and on scratching the arsonic acid separated and was purified as above described. The substance so obtained agreed in all of its properties with that obtained by the alternative method.

[1] THIS JOURNAL, **41**, 1589 (1919).
[2] *Ibid.*, **39**, 1441 (1917).
[3] *Ibid.*, **41**, 1590 (1919).

Subs., 0.1549: 10.6 cc. N (24.5°, 754 mm.).
Calc. for $C_{14}H_{15}O_4N_2As$: N, 8.00. Found: 7.79.

Sodium Salt.—On dissolving the arsonic acid in dil. sodium hydroxide solution until faintly alkaline to litmus and adding an equal volume of saturated sodium acetate solution, the sodium salt separated as glistening scales. After filtering and washing with 20% sodium acetate solution, and then with 85% alcohol, it was air-dried. The salt contained 4 molecules of water of crystallization. It is freely soluble in water and its dilute solutions do not give immediate precipitates with calcium or barium salts. With magnesia mixture a precipitate is formed only on heating, while heavy metal salts give insoluble precipitates at once.

Subs., air-dry, 0.9416: Loss, 0.1553 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{14}H_{14}O_4N_2AsNa.4H_2O$: H_2O, 16.22. Found: 16.49.
Subs., anhydrous, 0.1709: 11.4 cc. N (21.0°, 754 mm.).
Calc. for $C_{14}H_{14}O_4N_2AsNa$: N, 7.53. Found: 7.69.

Nitroso Compound, p-$H_2O_3AsC_6H_4N(NO)CH_2CONHC_6H_5$.—3 g. of the arsonic acid were suspended in 35 cc. of glacial acetic acid and treated slowly, with constant agitation, with 6 cc. of a 10% sodium nitrite solution. Only a portion of the substance dissolved and the mixture was heated to boiling, forming a clear, yellow solution. To complete the reaction 0.5 cc. of sodium nitrite solution was added and the mixture cooled, rosets of flat needles forming on scratching. After dilution with water to complete the separation the nitroso compound was filtered off and recrystallized from 50% alcohol. It separated as sheaves of long, flat, colorless needles containing one molecule of water of crystallization. The yield was 2.3 g. When rapidly heated to 185°, then slowly, it effervesces at 190–2°. It is very sparingly soluble in boiling water, readily in boiling 50% alcohol, quite easily in the cold in acetic acid, and somewhat sparingly so in cold 95% alcohol. The nitroso compound is turned brown by sulfuric acid but dissolves to a colorless solution; in the presence of phenol, however, a brown solution is obtained, changing rapidly to deep green.

Subs., air-dry, 0.4429: Loss, 0.0218 *in vacuo* at 80° over H_2SO_4.
Subs., 0.1023: 9.5 cc. N (24.0°, 761 mm.). Subs., 0.2979: $Mg_2As_2O_7$, 0.1154.
Calc. for $C_{14}H_{14}O_5N_3As.H_2O$: H_2O, 4.54; N, 10.58; As, 18.87. Found: H_2O, 4.92; N, 10.69; As, 18.70.

N-(Phenyl-4-arsonic Acid)-glycine-2'-toluidide, p-$H_2O_3AsC_6H_4NHCH_2CONHC_6H_4CH_3(o)$.—This substance was prepared as usual from chloroacetyl-*o*-toluidine, aided by the addition of sodium iodide. Purified by redissolving in a slight excess of dil. sodium hydroxide solution, warming, and reprecipitating with acetic acid the acid separated slowly as aggregates of flat, microscopic needles. Recrystallized from 50% alcohol it forms woolly masses of delicate needles which do not melt below 275° and are sparingly soluble in hot water and acetone. It is insoluble in the cold in 50% or 95% alcohol but appreciably so on boiling and readily so in hot acetic acid. It dissolves appreciably in cold methyl alcohol.

Subs., 0.2944: 9.9 cc. N (24.5°, 758 mm.).
Calc. for $C_{15}H_{17}O_4N_2As$: N, 7.69. Found: 7.69.

Sodium Salt.—On adding several volumes of alcohol to the neutral solution of the acid in dil. sodium hydroxide the sodium salt separates slowly as aggregates of long, narrow platelets which contain 2.5 molecules of water of crystallization.

Subs., air-dry, 0.5627: Loss, 0.0580 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{15}H_{16}O_4N_2AsNa.2.5H_2O$: H_2O, 10.44. Found: 10.31.
Subs., anhydrous, 0.2944: (Kjeldahl), 15.0 cc. 0.1 *N* HCl; $Mg_2As_2O_7$, 0.1175.
Calc. for $C_{15}H_{16}O_4N_2AsNa$: N, 7.26; As, 19.41. Found: N, 7.14; As, 19.27.

N-(Phenyl-4-arsonic Acid)-glycine-3'-toluidide.—The yield from 3.7 g. of chloroacetyl-*m*-toluidine[1] was 4 g. The crude product was recrystallized from 85% alcohol, separating as aggregates of long, thin plates. When rapidly heated it decomposes at about 285°, with preliminary darkening and softening. It is insoluble in boiling water or acetone, but is appreciably soluble in boiling methyl and ethyl alcohols and readily so in boiling acetic acid.

Subs., 0.1929: 13.3 cc. N (22.5°, 764 mm.). Subs., 0.3012: $Mg_2As_2O_7$, 0.1257.
Calc. for $C_{15}H_{17}O_4N_2As$: N, 7.70; As, 20.58. Found: N, 8.01; As, 20.16.

[1] *J. Biol. Chem.*, **21**, 108 (1915).

N-(Phenyl-4-arsonic Acid)-glycine-4'-toluidide.—This substance, obtained from chloroacetyl-*p*-toluidine, separates from the reaction mixture during the heating. Reprecipitated with acetic acid from its solution in sufficient hot dil. sodium hydroxide it separates as minute aggregates of short, flat, microscopic needles. It crystallizes from hot 50% alcohol, in which it is but sparingly soluble, as woolly masses of minute needles which do not decompose when heated up to 280°. It is practically insoluble in boiling water, and but sparingly so in boiling methyl or ethyl alcohol but is readily soluble in boiling acetic acid.

Subs., 0.1496: 9.8 cc. N (25.0°, 762 mm.).
Calc. for $C_{15}H_{17}O_4N_2As$: N, 7.69. Found: 7.53.

Sodium Salt.—When the acid is dissolved in hot, dil. sodium hydroxide solution and carefully neutralized with acetic acid the sodium salt separates on cooling as long, thin, curved, glistening needles, containing three molecules of water of crystallization. This salt is sparingly soluble in cold water and even less so in the presence of other sodium salts.

Subs., air-dry, 0.6936: Loss, 0.0856 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{15}H_{16}O_4N_2AsNa.3H_2O$: H_2O, 12.28. Found: 12.34.
Subs., anhydrous, 0.2989: (Kjeldahl), 15.3 cc. 0.1 *N* HCl; $Mg_2As_2O_7$, 0.1213.
Calc. for $C_{15}H_{16}O_4N_2AsNa$: N, 7.26; As, 19.41. Found: N, 7.17; As, 19.59.

N-(Phenyl-4-arsonic Acid)-glycine-α-naphthylamide, *p*-$H_2O_3AsC_6H_4NHCH_2CONHC_{10}H_7(\alpha)$.—Chloroacetyl-α-naphthylamine reacted smoothly, the product separating from the clear solution on scratching. It was best purified by precipitating a hot, dil. ammoniacal solution with acetic acid, forming aggregates of microscopic needles. It is practically insoluble in boiling water or 50% alcohol. When rapidly heated it darkens slightly, but does not melt up to 280°. On heating its solution in dil. sodium hydroxide the odor of α-naphthylamine is quickly noticeable owing to slight cleavage of the amide linking.

Subs., 0.1466: 9.0 cc. N (22.0°, 758 mm.). Subs., 0.3799: $Mg_2As_2O_7$, 0.1457.
Calc. for $C_{18}H_{17}O_4N_2As$: N, 7.00; As, 18.73. Found: N, 7.09; As, 18.51.

N-(Phenyl-4-arsonic Acid)-glycine-β-naphthylamide.—During the interaction of chloroacetyl-β-naphthylamine with sodium arsanilate in

the presence of sodium iodide the sparingly soluble chloro compound was gradually replaced by the reaction product, so that complete solution never occurred. After 4 hours' heating the mixture was diluted with water and treated with ammonia until definitely alkaline. The filtrate was warmed and treated with acetic acid, causing the precipitation of the arsonic acid as a crystalline powder. The yield from 4.4 g. of chloro compound was 5 g. For purification it was redissolved in dil. ammonia and reprecipitated with acetic acid, forming aggregates of microscopic needles which are practically insoluble in boiling water or 50% alcohol. When rapidly heated it decomposes at 285–6° to a red liquid.

Subs., 0.2154: (Kjeldahl), 10.6 cc. 0.1 *N* HCl.
Calc. for $C_{18}H_{17}O_4N_2As$: N, 7.00. Found: 6.90.

Sodium Salt.—When the acid is dissolved in warm, dil. sodium hydroxide solution and carefully neutralized with acetic acid the sparingly soluble sodium salt quickly separates as a lustrous, crystalline mass. Recrystallized from a small volume of 50% alcohol it forms aggregates of flat needles which contain approximately 4.5 molecules of water of crystallization.

Subs., air-dry, 0.8419: Loss, 0.1338 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{18}H_{16}O_4N_2AsNa.4.5H_2O$: H_2O, 16.11. Found: 15.89.
Subs., anhydrous, 0.3273: (Kjeldahl), 15.55 cc. 0.1 *N* HCl; $Mg_2As_2O_7$, 0.1214.
Calc. for $C_{18}H_{16}O_4N_2AsNa$: N, 6.64; As, 17.74. Found: N, 6.66; As, 17.90.

N-(Phenyl-4-Arsonic Acid)-glycinediphenylamide, p-$H_2O_3AsC_6H_4NHCH_2CON(C_6H_5)_2$.—On boiling equivalent amounts of sodium arsanilate, chloroacetyldiphenylamine,[1] and sodium iodide in 50% alcohol as in previous examples, an oily reaction product separated after several minutes. On continued boiling this redissolved and after about an hour the crystalline arsonic acid was deposited. The isolated product was dissolved in dil. ammonia, leaving an insoluble residue which was collected with boneblack and filtered off. On acidifying the filtrate to congo red with hydrochloric acid the diphenylamide was obtained and for final purification was redissolved in hot, very dil. ammonia and precipitated with acetic acid. The

[1] H. Frerichs, *Chem. Zentr.*, **74**, 103–4 (1903).

arsonic acid separated as long, thin, microscopic leaflets which contained one molecule of water of crystallization. The anhydrous substance decomposes at 271–2° with slight preliminary softening and is very sparingly soluble in boiling water or 50% alcohol.

Subs., air-dry, 0.6797: Loss, 0.0267 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{20}H_{19}O_4N_2As.H_2O$: H_2O, 4.06. Found: 3.93.
Subs., anhydrous, 0.1780: (Kjeldahl), 8.3 cc. 0.1 *N* HCl. Subs., 0.2355: $Mg_2As_2O_7$, 0.0853.
Calc. for $C_{20}H_{19}O_4N_2As$: N, 6.57; As, 17.58. Found: N, 6.53; As, 17.48.

N-(Phenyl-4-arsonic Acid)-glycine-4'-chloroanilide, p-$H_2O_3AsC_6H_4NHCH_2CONHC_6H_4Cl$ (p-).—Precipitated from the hot solution of its sodium salt with acetic acid, the substance obtained from chloroacetyl-p-chloroaniline quickly separates as toothed, microscopic leaflets which are often cross-shaped. It does not melt below 280° and is almost insoluble in boiling water or 50% alcohol. The sodium salt is easily salted out from its solutions by sodium acetate.

Subs., 0.3058: (Kjeldahl), 15.5 cc. 0.1 *N* HCl; $Mg_2As_2O_7$, 0.1238.
Calc. for $C_{14}H_{14}O_4N_2ClAs$: N, 7.29; As, 19.49. Found: N, 7.10; As, 19.54.

N-(Phenyl-4-arsonic Acid)-glycine-4'-iodoanilide.—(From chloroacetyl-p-iodoaniline.[1]) When added to the hot solution of its sodium salt, acetic acid causes the separation of the arsonic acid as broad, minute needles which do not melt below 275° and are practically insoluble in boiling water.

Subs., 0.2066: (Kjeldahl), 8.45 cc. 0.1 *N* HCl.
Calc. for $C_{14}H_{14}O_4N_2IAs$: N, 5.89. Found: 5.73.

Sodium Salt.—When an aqueous suspension of the acid was neutralized with sodium hydroxide and treated with sodium acetate solution the sodium salt crystallized as glistening leaflets. Recrystallized from 85% alcohol the salt separated in rosets of needles which contained 3.5 molecules of water of crystallization.

Subs., air-dry, 0.4029: Loss, 0.0448 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{14}H_{13}O_4N_2IAsNa.3.5H_2O$: H_2O, 11.23. Found: 11.12.
Subs., anhydrous, 0.2946: (Kjeldahl), 11.65 cc. 0.1 *N* HCl; $Mg_2As_2O_7$, 0.0922.
Calc. for $C_{14}H_{13}O_4N_2IAsNa$: N, 5.63; As, 15.05. Found: N, 5.54; As, 15.11.

[1] This Journal, **39**, 1441 (1917).

N-(Phenyl-4-arsonic Acid)-glycine-4'-nitroanilide, *p*-$H_2O_3AsC_6H_4NHCH_2CONHC_6H_4NO_2(p)$.—In this case the sparingly soluble chloroacetyl-*p*-nitraniline[1] was gradually replaced by the reaction product. The crude substance was suspended in a large volume of hot water and treated with a slight excess of ammonia. The hot, filtered solution was then treated with an excess of hydrochloric acid, causing the precipitation of the arsonic acid as long, thin, faintly yellow needles. Acetic acid is not a sufficiently strong acid completely to displace the nitro acid from its ammonium salt, and as this is also sparingly soluble in water it contaminates the free arsonic acid unless a mineral acid is used as precipitant. The yield from 14.5 g. of chloroacetyl-*p*-nitraniline was 18.5 g. The arsonic acid is practically insoluble in boiling water or 50% alcohol and does not melt below 285°.

Subs., 0.1217: 11.25 cc. N (27.0°, 762 mm.). Subs., 0.3110: $Mg_2As_2O_7$, 0.1212.
Calc. for $C_{14}H_{14}O_6N_3As$: N, 10.63; As, 18.98. Found: N, 10.55; As, 18.81.

N-(Phenyl-4-arsonic Acid)-glycine-4'-aminoanilide, *p*-$H_2O_3AsC_6H_4NHCH_2CONHC_6H_4NH_2(p)$.—This substance could not be made directly from *p*-amino-chloroacetanilide because of the inaccessibility of the latter compound. It is also probable that the presence of the amino group would prevent a smooth reaction in the desired sense. The substance was therefore obtained indirectly, both by the saponification of the acetyl compound described below and by reduction[2] of the nitro compound just mentioned. From the standpoint of yield the latter method is to be preferred. The former, however, is more rapid.

16 g. of (phenyl-4-arsonic acid)-glycine-4'-nitroanilide were dissolved in 50 cc. of hot 2 *N* sodium hydroxide solution, whereupon the sodium salt suddenly separated in finely divided form. This suspension was added to a bottle containing a slime of ferrous hydroxide prepared by precipitating a cold, saturated solution of 80 g. of ferrous sulfate with sufficient 25% sodium hydroxide solution to render the mixture strongly alkaline to litmus after thorough shaking. The reduction occurred at once in the cold, as evidenced by the change in

[1] *J. Biol. Chem.*, **21**, 112 (1915).
[2] This Journal, **40**, 1580 (1918).

color of the iron hydroxide. The bottle was immediately stoppered and shaken vigorously for several minutes and the mixture then poured on to a large suction funnel. On acidifying the filtrate with acetic acid a copious precipitate of the crude amino acid separated. This was filtered off, redissolved in hot, dil. ammonia, and reprecipitated with acetic acid. For final purification it was dissolved in a considerable volume of warm, 10% sulfuric acid, treated with bone-black, and sodium acetate solution added to the warm filtrate until it no longer turned congo red paper blue. The amino acid separated in almost quantitative yield as colorless, microscopic needles or platelets. When heated rapidly it decomposes at 253–4° with preliminary darkening. It is practically insoluble in boiling water or 50% alcohol and dissolves in dil. hydrochloric acid, the hydrochloride being readily salted out by an excess of the acid. The acid solution is readily diazotized, coupling with R-salt to form a red dye.

Subs., 0.1345: 13.25 cc. N (24.5°, 760 mm.). Subs., 0.1553: $Mg_2As_2O_7$, 0.0654.
Calc. for $C_{14}H_{16}O_4N_3As$: N, 11.51; As, 20.52. Found: N, 11.31; As, 20.32.

Owing to the comparative stability of the anilide linking in *N*-(phenyl-4-arsonic acid)-glycine-4′-acetaminoanilide (see below), it was possible to remove the acetyl group in this compound by short boiling with hydrochloric acid. A partial rupture of the anilide linking could not be avoided, however, and consequently a good yield of the amino compound was not obtained. The mechanical difficulties arising from the sparing solubilities of the hydrochlorides of both the acetamino and amino acids were overcome by the addition of alcohol to the hydrolysis mixture, and the following conditions were found to permit of a rapid and smooth reaction.

5 g. of *N*-(phenyl-4-arsonic acid)-glycine-4′-acetaminoanilide were suspended in a mixture of 45 cc. of 1:1 hydrochloric acid and 15 cc. of alcohol. On boiling the solution under a reflux condenser the arsonic acid gradually dissolved. After 10 minutes' boiling the clear solution was chilled, causing the precipitation of the hydrochloride of the aminoanilide. The mixture was diluted with water, treated with ammonia in slight excess, heated on the water bath, and acidified with acetic acid, the amino compound separating as minute micro-

scopic needles agreeing in all properties with the substance obtained by the reduction of the nitro compound. The yield was 2 g.

Subs., 0.1539: 15.6 cc. N (22.0°, 760 mm.). Subs., 0.2902: $Mg_2As_2O_7$, 0.1222.
Calc. for $C_{14}H_{16}O_4N_3As$: N, 11.51; As, 20.52. Found: N, 11.73; As, 20.30.

N-(Phenyl-4-arsonic Acid)-glycine-4'-acetaminoanilide.—Owing to the sparing solubility of *p*-chloroacetylamino-acetanilide[1] the reaction mixture remained a thick paste in this case. After 4 hours' heating, however, the suspended material was changed in appearance and finally became completely soluble in dil. sodium hydroxide solution. The substance was purified in the usual manner. From hot, dilute solutions of the sodium salt acetic acid causes the gradual separation of the acid as aggregates of microscopic needles which are practically insoluble in boiling water or 50% alcohol and do not melt below 285°. 22 g. of arsanilic acid yielded 29 g. of the new arsonic acid.

Subs., 0.1439: 13.0 cc. N (22.5°, 763 mm.).
Calc. for $C_{16}H_{18}O_5N_3As$: N, 10.32. Found: 10.48.

Sodium Salt.—On dissolving the arsonic acid in sodium hydroxide solution to a slight alkaline reaction and adding alcohol until crystallization begins, the salt gradually separates as minute, lustrous platelets which slowly lose part of their water of crystallization on exposure to the air. A portion recrystallized from hot water and air-dried also gave uncertain figures for water of crystallization.

Subs., air-dry, 0.4421: Loss, 0.0379 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{16}H_{17}O_5N_3AsNa.2H_2O$: $2H_2O$, 7.75; $2\frac{1}{2}$ H_2O, 9.49. Found: 8.57.
Subs., anhydrous, 0.1324: 11.4 cc. N (21.5°, 761 mm.). Subs., 0.3218: $Mg_2As_2O_7$, 0.1140.
Calc. for $C_{16}H_{17}O_5N_3AsNa$: N, 9.79; As, 17.47. Found: N, 9.99; As, 17.09.

N-(Phenyl-4-arsonic Acid)-glycine-4'-uramino-anilide, *p*-$H_2O_3AsC_6H_4NHCH_2CONHC_6H_4NHCONH_2$(*p'*-).—(From *p*-chloroacetylamino-phenyl urea.[2]) After one hour's boiling the arsonic acid suddenly separated from the clear solution. After an additional hour's heating the substance was filtered off and purified over the sodium salt described below. The free acid was precipitated by acetic acid from a

[1] This Journal, **39**, 1455 (1917).
[2] *Ibid.*, **39**, 1456 (1917).

hot solution of the salt as pale brown, microcrystalline aggregates which contain $\frac{1}{2}$ molecule of water of crystallization. It is very sparingly soluble in boiling water, 50% alcohol, or methyl alcohol. When rapidly heated the anhydrous substance darkens above 200° and decomposes at 230°.

Subs., air-dry, 1.1959: Loss, 0.0299 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{15}H_{17}O_5N_4As.0.5H_2O$: H_2O, 2.16. Found: 2.50.
Subs., anhydrous, 0.1555: 19.0 cc. N (27.0°, 759 mm.).
Calc. for $C_{15}H_{17}O_5N_4As$: N, 13.73. Found: 13.89.

Sodium Salt.—A neutral solution of the crude acid as obtained above in a small volume of dil. sodium hydroxide solution was treated with an equal volume of saturated sodium acetate solution. On standing in the refrigerator the salt crystallized as a thick mass of colorless, microscopic needles. These were filtered off, washed with 85% alcohol, and recrystallized by dissolving in a small volume of warm water and treating the solution with an equal volume of alcohol. The filtered product was washed with 50% alcohol. Starting with 22 g. of arsanilic acid the yield was 24 g. The salt contains 4 molecules of water of crystallization.

Subs., air-dry, 0.5620: Loss, 0.0822 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{15}H_{16}O_5N_4AsNa.4H_2O$: H_2O, 14.35. Found: 14.63.
Subs., anhydrous, 0.1311: 14.8 cc. N (20.5°, 756 mm.). Subs., 0.3050: $Mg_2As_2O_7$, 0.1087.
Calc. for $C_{15}H_{16}O_5N_4AsNa$: N, 13.02; As, 17.42. Found: N, 13.07; As, 17.20.

N-(Phenyl-4-arsonic Acid)-glycine-4'-methyl-5'-uramino-anilide, *p*-$H_2O_3AsC_6H_4NHCH_2CONHC_6H_3(CH_3)NHCONH_2$ (*p'*, *m'*-).—Starting with 18.4 g. of 2-methyl-5-chloroacetylamino-phenyl urea,[1] the crude acid which separated during the heating was dissolved in dil. sodium hydroxide solution, the filtrate diluted to about 500 cc., heated, and then acidified with acetic acid. The new arsonic acid separated as aggregates of microscopic platelets and hairs which rendered filtration difficult. The yield was 22 g. The substance contains approximately $\frac{1}{2}$ molecule of water of crystallization. When rapidly heated the anhydrous acid decomposes at 257–8° with preliminary darkening and sintering and is practically insoluble in the usual solvents.

[1] This Journal, **39**, 1464 (1917).

Subs., air-dry, 0.5964: Loss, 0.0103 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{16}H_{19}O_5N_4As.0.5H_2O$: H_2O, 2.09. Found: 1.73.
Subs., anhydrous, 0.1413: 16.3 cc. N (23.0°, 758 mm.). Subs., 0.3553: $Mg_2As_2O_7$, 0.1305
Calc. for $C_{16}H_{19}O_5N_4As$: N, 13.26; As, 17.76. Found: N, 13.27; As, 17.72.

Sodium Salt.—A suspension of the acid in a little hot water was dissolved by adding just enough sodium hydroxide solution and treated with alcohol until just turbid. On rubbing and letting stand the salt slowly separated as a crystalline powder. A similar recrystallization was necessary to obtain an entirely pure product. The air-dried salt contained approximately 3.5 molecules of water of crystallization and was readily soluble in water. It can be recrystallized from hot water and is slowly salted out from strong solutions by sodium acetate.

Subs., air-dry, 0.7448: Loss, 0.0898 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{16}H_{18}O_5N_4AsNa.3.5H_2O$: H_2O, 12.48. Found: 12.05.
Subs., anhydrous, 0.1518: 16.4 cc. N (21.5°, 768 mm.). Subs., 0.3272: $Mg_2As_2O_7$, 0.1174.
Calc. for $C_{16}H_{18}O_5N_4AsNa$: N, 12.61; As, 16.88. Found: N, 12.66; As, 17.31.

N-(Phenyl-4-arsonic Acid)-glycine-3'-oxamylaminoaniline, *p*-$H_2O_3AsC_6H_4NHCH_2CONHC_6H_4NHCOCONH_2$(*m'*-).—5.2 g. of *m*-chloroacetylamino-oxanilamide[1] were boiled 6 hours with the usual reaction mixture. The precipitate was then filtered off, suspended in about a liter of water and treated with a slight excess of ammonia. The insoluble material was collected by adding boneblack and the filtrate heated nearly to boiling. The addition of acetic acid caused the gradual deposition of the arsonic acid as slightly purplish, microcrystalline aggregates. The yield was 4.4 g. The acid is almost insoluble in boiling water and very sparingly soluble in boiling 50% alcohol. When rapidly heated it gradually darkens and partly decomposes, but does not melt up to 280°.

Subs., 0.1514: 17.0 cc. N (23.0°, 757 mm.). Subs., 0.2985: $Mg_2As_2O_7$, 0.1076.
Calc. for $C_{16}H_{17}O_6N_4As$: N, 12.85; As, 17.19. Found: N, 12.90; As, 17.39.

N-(Phenyl-4-arsonic Acid)-glycine-4'-oxamylaminoanilide.—Owing to the insolubility of *p*-chloroacetylamino-oxanilamide[2] the heating in this case was continued for about 7 hours, in spite of which the reaction

[1] This Journal, **39**, 1452 (1917).
[2] *Ibid.*, **39**, 1461 (1917).

was very incomplete. The filtered solid was suspended in a large volume of water and the mixture treated with a slight excess of ammonia. After thorough disintegration of the insoluble residue the mixture was filtered and the solution acidified with acetic acid, causing the immediate precipitation of the arsonic acid as arborescent aggregates of microscopic needles. The yield from 4.8 g. of chloro compound was only 1.1 g. The substance is practically insoluble in all neutral solvents and does not melt below 280°.

Subs., 0.1812: 20.0 cc. N (20.5°, 755 mm.). Subs., 0.2847: $Mg_2As_2O_7$, 0.1002. Calc. for $C_{16}H_{17}O_6N_4As$: N, 12.85; As, 17.19. Found: N, 12.75; As, 16.99.

N-(Phenyl-4-arsonic Acid) glycyl-2-aminophenol, (*N-(Phenyl-4-arsonic Acid)-glycine-2'-hydroxyanilide*), *p*-$H_2O_3AsC_6H_4NHCH_2CONHC_6H_4OH$(*o*-).—The halogen in the chloroacetylamino-phenols is extremely labile, so that these substances react with the amino group so readily as to render unnecessary the addition of sodium iodide as in the previously described syntheses. In the case of *o*-chloroacetylamino-phenol this reactivity causes a complication, since in the presence of alkalies or the sodium salts of weak acids it readily undergoes *ortho* condensation with the formation of *o*-amino-phenoxyacetic anhydride.[1] This reaction consequently paralleled the glycine formation, but in spite of this the desired arsonic acid was obtained, although in rather poor yield.

4.4 g. of arsanilic acid were dissolved in 20 cc. of *N* sodium hydroxide solution and boiled under an air-condenser with 3.8 g. of *o*-chloroacetyl-amino-phenol[2] for one hour, the oil which first formed gradually disappearing. On cooling the reaction product separated as an oil, but on acidifying to congo red with hydrochloric acid and scratching, it solidified almost completely. The product was collected and digested with dil. ammonia, leaving 1.5 g. of a crystalline residue, which, both by melting point (170–5°) and analysis, was shown to be *o*-amino-phenoxyacetic anhydride. On warming the ammoniacal filtrate from this by-product and acidifying slightly with hydrochloric acid, 1.7 g. of the new arsonic acid separated on cooling as lustrous crystals. When rapidly heated the substance melts and decomposes at 190°

[1] Aschan, *Ber.*, **20**, 1524 (1887).

[2] This Journal, **41**, 458 (1919).

with preliminary darkening. It is soluble in cold 95% alcohol and is also easily soluble in boiling water, from which it separates on cooling as long, narrow, glistening leaflets. An alkaline solution couples readily with diazotized sulfanilic acid, yielding an orange colored solution.

Subs., 0.1496: (Kjeldahl), 8.23 cc. 0.1 *N* HCl. Subs., 0.2221: $Mg_2As_2O_7$, 0.0958.
Calc. for $C_{14}H_{15}O_5N_2As$: N, 7.66; As, 20.48. Found: N, 7.70; As, 20.82.

A better yield of the arsonic acid was obtained by using two molecules of arsanilic acid instead of one molecule of sodium arsanilate. In this case longer boiling was necessary. 21 g. of *o*-chloroacetylaminophenol and 50 g. of arsanilic acid (2 mols.) in 220 cc. of water were boiled under a reflux condenser for 4 hours. A clear solution was quickly obtained but this gradually darkened. At the end 50 cc. of 10% hydrochloric acid were added and on cooling and scratching the arsonic acid gradually crystallized. After purification as described above it agreed in all its properties with the product isolated by the first method. The yield was 17 g.

Subs., 0.1545: (Kjeldahl), 8.6 cc. 0.1 *N* HCl. Subs., 0.3010: $Mg_2As_2O_7$, 0.1254.
Calc. for $C_{14}H_{15}O_5N_2As$: N, 7.66; As, 20.48. Found: N, 7.76; As, 20.11.

N-(Phenyl-4-arsonic Acid)glycyl-3-aminophenol (*N-(Phenyl-4-arsonic Acid)-glycine-m'-hydroxyanilide*).—Owing to the special importance of this substance as the starting material for the preparation of arsenophenylglycine-bis-*m'*-hydroxyanilide, it will be published in connection with this arseno compound in another place.

N-(Phenyl-4-arsonic Acid)-glycine-2'-methyl-5'-hydroxyanilide.—When a solution of 12.4 g. of arsanilic acid in 57 cc. of *N* aqueous sodium hydroxide was boiled with 11.3 g. of 4-methyl-5-chloroacetylamino-phenol[1] a clear solution was obtained in a few minutes and deposition of the new arsonic acid began in about 20 minutes. Heating was continued on the water bath for a total of one hour, and after letting cool, the mixture was acidified to congo red with hydrochloric acid and filtered. The crude product was suspended in water, made just alkaline with sodium carbonate, and treated with boneblack after neutralizing with acetic acid. The filtrate, heated almost to

[1] This Journal, **41**, 459 (1919).

the boiling point and treated with acetic acid, soon deposited the arsonic acid as glistening, pink platelets and microscopic prisms. The yield was 9.0 g., decomposing at about 220–5°, with preliminary darkening. It is very sparingly soluble in boiling water, alcohol, methyl alcohol, or acetic acid. In alkaline solution it couples with diazotized sulfanilic acid yielding a redder color than do similar compounds in which the position *para* to the hydroxy group is unoccupied.

Subs., 0.1308: (Kjeldahl), 7.0 cc. 0.1 *N* HCl. Subs., 0.3134: $Mg_2As_2O_7$, 0.1267. Calc. for $C_{15}H_{17}O_5N_2As$: N, 7.37; As, 19.72. Found: N, 7.50; As, 19.49.

N-(Phenyl-4-arsonic Acid)-glycine-4'-methyl-5'-hydroxyanilide.—The product from 20 g. of 2-methyl-5-chloroacetylamino-phenol[1] was worked up as in the case of the preceding compound, but as the analysis showed it to be impure it was converted into the sodium salt. The salt obtained by the addition of several volumes of alcohol to the neutral solution of the acid in sodium hydroxide solution was washed with 85% alcohol, and converted into the acid by precipitating from hot water with acetic acid. The arsonic acid forms prisms and branched leaflets which are very sparingly soluble in the usual solvents. The yield was 10.6 g. When rapidly heated to 255°, then slowly, the substance gradually darkens and softens, melting at 258° with decomposition. In alkaline solution it couples readily with diazotized sulfanilic acid.

Subs., 0.1596: (Kjeldahl), 12.1 cc. 0.1 *N* HCl. Subs., 0.3030: $Mg_2As_2O_7$, 0.1228. Calc. for $C_{15}H_{17}O_5N_2As$: N, 7.37; As, 19.72. Found: N, 7.58; As, 19.57.

N-(Phenyl-4-arsonic Acid)glycyl-4'-aminophenol, (N-(Phenyl-4-arsonic Acid)-glycine-4'-hydroxyanilide).—After about 15 minutes the mixture (from 33 g. of arsanilic acid and 28 g. of *p*-chloroacetylamino-phenol[2]) set to an almost solid mass of lustrous crystals and the heating was continued for 45 minutes longer on the water bath. The somewhat colored product was suspended in a moderate volume of hot water and completely dissolved by the addition of dil. sodium hydroxide solution. After careful neutralization with acetic acid the hot solution was decolorized with boneblack and the clear, hot filtrate acidified with acetic acid, yielding the pure arsonic acid as faintly pink, glistening platelets in a yield of 35 g. When rapidly heated the arsonic acid

[1] This Journal, **41**, 459 (1919).
[2] *Ibid.*, **39**, 1442 (1917).

darkens and melts to a black tar at 255–60°. It is almost insoluble in boiling water, 50% alcohol, 50% acetic acid, or methyl alcohol.

Subs., 0.2673: (Kjeldahl), 14.25 cc. 0.1 *N* HCl.
Calc. for $C_{14}H_{15}O_5N_2As$: N, 7.66. Found: 7.47.

Sodium Salt.—This was readily obtained from a neutral solution of the acid in dil. sodium hydroxide on treatment with saturated sodium acetate solution. Recrystallized from hot water, the salt formed lustrous platelets which contained 4.5 molecules of water of crystallization. It is fairly easily soluble in cold water.

Subs., air-dry, 0.5730: Loss, 0.0967 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{14}H_{14}O_5N_2AsNa.4.5H_2O$: H_2O, 17.26. Found: 16.87.
Subs., anhydrous, 0.3096: (Kjeldahl), 16.25 cc. 0.1 *N* HCl; $Mg_2As_2O_7$, 0.1245.
Calc. for $C_{14}H_{14}O_5N_2AsNa$: N, 7.22; As, 19.30. Found: N, 7.36; As, 19.41.

N-(Phenyl-4-arsonic Acid)-glycine-p'-anisidide, p-$H_2O_3AsC_6H_4NHCH_2CONHC_6H_4OCH_3(p)$-.—This substance was readily obtained by the usual method from chloroacetyl-*p*-anisidine in the presence of sodium iodide. The arsonic acid is precipitated by acetic acid from its hot solution in dil. sodium hydroxide as lustrous leaflets which darken and soften above 230° when rapidly heated. It is practically insoluble in boiling water or boiling 50% alcohol, and gives a flesh-colored solution in conc. sulfuric acid.

Subs., 0.1575: 10.2 cc. N (23.5°, 759 mm.). Subs., 0.3422: $Mg_2As_2O_7$, 0.1368.
Calc. for $C_{15}H_{17}O_5N_2As$: N, 7.37; As, 19.71. Found: N, 7.45; As, 19.29.

N-(Phenyl-4-arsonic Acid)glycyl-1-amino-2-naphthol, p-$H_2O_3AsC_6H_4NHCH_2CONHC_{10}H_6OH(1,2)$-.—The ease with which 1-chloroacetyl-amino-2-naphthol[1] reacts in the presence of alkali, alkali carbonates, or salts of weak acids to form the anhydride of 1-amino-2-naphthoxy-acetic acid interfered greatly with the preparation of the above arsonic acid, a fact which recalls the experience with *o*-chloroacetylamino-phenol. Of all the methods tested, the best results were given by the following procedure:

22 g. of arsanilic acid in 100 cc. of *N* sodium hydroxide solution, 20 g. of sodium iodide, 24 g. of chloroacetylamino-phenol and 100 cc. of alcohol were boiled for one hour, a large proportion of the chloroacetyl

[1] THIS JOURNAL, **41**, 460 (1919).

compound changing to the sparingly soluble anhydride. After dilution with water, the mixture was made ammoniacal and the insoluble anhydride (m. p. 215°) filtered off. The filtrate was acidified strongly with acetic acid and on standing in the refrigerator the arsonic acid gradually separated, contaminated with unchanged arsanilic acid. The deposited substance was redissolved in dil. ammonia, treated with bone-black, and the hot filtrate acidified with hydrochloric acid, the new compound separating at once as a heavy powder consisting of aggregates of microscopic plates and prisms which contained two molecules of water of crystallization. The yield was 6.5 g. The anhydrous substance decomposes at 189–91° with preliminary darkening and softening. Although very difficultly soluble in the cold it dissolved appreciably in boiling alcohol or acetic acid. It is more easily soluble in methyl alcohol and almost insoluble in water.

Subs., air-dry, 0.5668: Loss, 0.0453 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{18}H_{17}O_5N_3As.2H_2O$: H_2O, 7.96. Found: 7.99.
Subs., anhydrous, 0.1970: (Kjeldahl), 9.55 cc. 0.1 *N* HCl. Subs., 0.3666: $Mg_2As_2O_7$, 0.1386.
Calc. for $C_{18}H_{17}O_5N_2As$: N, 6.73; As, 18.02. Found: N, 6.79; As, 18.24.

(*N-(Phenyl-4-arsonic Acid)-glycine-4',1'-hydroxynaphthalide*), *N-(Phenyl-4-arsonic Acid)glycyl-4-amino-1-naphthol.*—4.4 g. of arsanilic acid were treated in the usual manner with 4.8 g. of 4-chloroacetylamino-1-naphthol[1] in 50% alcohol in the presence of sodium iodide for 4 hours. The deeply colored solution yielded the new arsonic acid on scratching and dilution with water. After filtration and washing with acetone in order to remove most of the color, the crude residue was dissolved in hot 20% sodium acetate solution and then treated with an equal volume of saturated sodium acetate solution. On cooling the sodium salt separated as glistening plates, and was recrystallized from a small volume of water. The yield was 4 g., containing 5.5 molecules of water of crystallization.

Subs., air-dry, 0.9469: Loss, 0.1704 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{18}H_{16}O_5N_2AsNa.5.5H_2O$: H_2O, 18.43. Found: 17.99.
Subs., anhydrous, 0.2997: (Kjeldahl) 13.65 cc. 0.1 *N* HCl; $Mg_2As_2O_7$, 0.1060.
Calc. for $C_{18}H_{16}O_5N_2AsNa$: N, 6.39; As, 17.11. Found: N, 6.38; As, 17.07.

[1] This Journal, **41**, 460 (1919).

On treating a hot, dilute solution of the sodium salt with acetic acid, the *free acid* separates slowly as microscopic crystals which contain approximately 1.5 molecules of water of crystallization. The anhydrous substance darkens above 200° and decomposes at 240–2°. It is practically insoluble in boiling water and but sparingly so in 50% or 95% alcohol. It is readily soluble in hot methyl alcohol.

Subs., air-dry, 0.6011: Loss, 0.0405 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{18}H_{17}O_5N_2As.1.5H_2O$: H_2O, 6.10. Found: 6.74.
Subs., anhydrous, 0.1452: 8.7 cc. N (27.5°, 760 mm.).
Calc. for $C_{18}H_{17}O_5N_2As$: N, 6.73. Found: 6.81.

N-(Phenyl-4-arsonic Acid)glycyl-3-amino-4,6-dichlorophenol.—In the reaction between 2,4-dichloro-5-chloroacetylamino-phenol[1] and sodium arsanilate by the usual sodium iodide-50% alcohol method a clear solution quickly formed from which the reaction product suddenly separated. The solid was suspended in a large volume of water to prevent separation of the somewhat difficultly soluble sodium salt and dil. sodium hydroxide solution added until all but an amorphous substance had dissolved. This was collected with boneblack and the filtrate heated and acidified with acetic acid. A repetition of this process removed a further small quantity of amorphous material, 26 g. of the original chloroacetyl compound yielding 21 g. of the pure product. The new arsonic acid forms flat, colorless microscopic needles which begin to darken above 220° but melt and decompose at about 280°. It is practically insoluble in boiling water or 50% alcohol.

Subs., 0.2538: (Kjeldahl), 12.02 cc. 0.1 *N* HCl. Subs., 0.2274: $Mg_2As_2O_7$, 0.0809.
Calc. for $C_{14}H_{13}O_5N_2Cl_2As$: N, 6.44; As, 17.23. Found: N, 6.64; As, 17.16.

N-(Phenyl-4-arsonic Acid)glycyl-3-amino-6-bromophenol.—16.5 g. of arsanilic acid and 20 g. of 2-bromo-5-chloroacetylamino-phenol[2] yielded smoothly 23 g. of recrystallized reaction product by the sodium iodide-50% alcohol method. Acetic acid precipitates the arsonic acid from hot solutions of its salts as glistening leaflets which decompose at 255° with preliminary sintering. It is very sparingly soluble in boiling water, 50% alcohol, or methyl alcohol.

[1] This Journal, **41**, 461 (1919).
[2] *Ibid.*, **41**, 464 (1919).

Subs., 0.1421: 7.6 cc. N (21.5°, 763 mm.).
Calc. for $C_{14}H_{14}O_5N_2BrAs$: N, 6.29. Found: 6.23.

N-(Phenyl-4-arsonic Acid)glycyl-4-amino-pyrocatechol, (N-(Phenyl-4-arsonic Acid)-glycine-3′,4′-dihydroxyanilide).—A solution of 33 g. of arsanilic acid in 150 cc. of *N* sodium hydroxide solution was boiled for one hour with 31 g. of 4-chloroacetylamino-pyrocatechol,[1] the reaction product partly separating from the deeply colored solution. After cooling the very dark crystalline mass was filtered off, dissolved in dil. sodium hydroxide solution, and carefully neutralized with acetic acid. After treating the almost black solution with boneblack and acidifying the still deeply colored filtrate with acetic acid, the arsonic acid slowly separated as colored, microscopic leaflets. A repetition of the purification process removed most of the color, the product as finally obtained forming faintly pink, glistening leaflets. The yield was only 15 g., considerable loss having occurred during purification. When rapidly heated the substance blackens above 200° and decomposes at about 260–5°. It dissolves with difficulty in boiling water, separating on cooling as long, narrow, lustrous plates. It is very sparingly soluble in hot alcohol or methyl alcohol, more readily in hot 50% alcohol. A solution of the acid in an excess of dil. sodium hydroxide solution rapidly turns deep orange in color, while ferric chloride added to an aqueous solution of the acid causes a bluish purple coloration.

Subs., 0.1653: (Kjeldahl), 8.67 cc. 0.1 *N* HCl. Subs., 0.3105: $Mg_2As_2O_7$, 0.1274.
Calc. for $C_{14}H_{15}O_6N_2As$: N, 7.33; As, 19.61. Found: N, 7.34; As, 19.80.

N-(Phenyl-4-arsonic Acid)glycyl-anthranilic Acid, p-$H_2O_3AsC_6H_4NHCH_2CONHC_6H_4CO_2H(o)$-.—Owing to the great reactivity of the chlorine atom in chloroacetyl-anthranilic acid the reaction between this compound and sodium arsanilic did not go entirely in the desired sense, so that the yield of the complex arsonic acid was unusually poor. The most satisfactory results were obtained by the use of the ester of chloroacetyl-anthranilic acid and subsequent saponification of the resulting ester arsonic acid.

30 g. of *N*-(phenyl-4-arsonic acid)glycyl-anthranilic ethyl ester (see below) were dissolved in a definite excess of 10% sodium hydroxide

[1] This Journal, **41**, 468 (1919).

solution and allowed to stand at room temperature for several hours. Heating the solution was found not only to saponify the ester group but also to cleave the amide linking with consequent formation of anthranilic acid. Acidification caused the separation of a gum which rapidly crystallized and was redissolved in hot dil. sodium acetate solution and treated hot with an excess of hydrochloric acid. The arsonic acid separated at once as characteristic octahedra. The yield was 25 g. On rapid heating it decomposes at 230–5° with preliminary softening and darkening and is practically insoluble in boiling water, but appreciably in boiling methyl or ethyl alcohol or 50% alcohol.

Subs., 0.3056: (Kjeldahl), 15.4 cc. 0.1 *N* HCl; $Mg_2As_2O_7$, 0.1195.
Calc. for $C_{15}H_{15}O_6N_2As$: N, 7.11; As, 19.02. Found: N, 7.06; As, 18.88.

N-(Phenyl-4-arsonic Acid)glycyl-anthranilic Ethyl Ester, *p*-$H_2O_3AsC_6H_4NHCH_2CONHC_6H_4CO_2C_2H_5$(*o*-).—25 g. of chloroacetyl-anthranilic ethyl ester,[1] in the presence of sodium iodide yielded 31 g. of crude product. Recrystallized from 50% alcohol the ester separates as rosets of delicate needles which do not decompose below 280°. It is difficultly soluble in cold methyl alcohol, more readily on heating and also dissolved in hot 50% or 95% alcohol.

Subs., 0.1485: 8.9 cc. N (25.5°, 755 mm.).
Calc. for $C_{17}H_{19}O_6N_2As$: N, 6.64. Found: 6.81.

N-(Phenyl-4-arsonic Acid)glycyl-N-methylanthranilic Acid, *p*-$H_2O_3AsC_6H_4NHCH_2CON(CH_3)C_6H_4CO_2H$(*o*-).—Although the reaction between sodium arsanilate and chloroacetyl-methylanthranilic acid[2] apparently proceeded partly in the desired sense, it was difficult to isolate a pure product. As in the case of the anthranilic acid compound the substance was found to be more easily prepared over the ester. For this purpose 25 g. of arsanilic acid dissolved in 115 cc. of *N* sodium hydroxide solution, 29 g. of chloroacetyl-methylanthranilic ethyl ester,[3] 22 g. of sodium iodide, and 115 cc. of alcohol were boiled under a reflux condenser for 4 hours. As attempts to obtain a crystalline reaction product failed the ester was not isolated. Instead the alco-

[1] This Journal, **41**, 469 (1919).
[2] *Ibid.*, **41**, 470 (1919).
[3] *Ibid.*, **41**, 470 (1918).

hol was allowed to boil off and the residue diluted with water. After the oil which was formed had settled, the aqueous layer was decanted and the oil again shaken with a small volume of water which was then also decanted. The crude ester so obtained was dissolved in 150 cc. of 10% sodium hydroxide solution, filtered, and allowed to stand 4 hours at room temperature. The alkaline solution was then acidified to congo red with hydrochloric acid, causing the separation of the acid as a gum which gradually solidified. This was purified by again dissolving in dil. alkali and acidifying with hydrochloric acid until faintly acid to congo red. The resulting cloudy solution gradually deposited the acid as microscopic aggregates of needles or short, flat plates. The yield was 16 g. When rapidly heated the acid darkens and decomposes at 230°. It is very difficultly soluble in boiling water or boiling methyl alcohol, but fairly readily so in hot 50% alcohol, from which it can be recrystallized.

Subs., 0.3213: (Kjeldahl), 15.7 cc. 0.1 *N* HCl. Subs., 0.3256: $Mg_2As_2O_7$, 0.1245.
Calc. for $C_{16}H_{17}O_6N_2As$: N, 6.86; As, 18.36. Found: N, 6.85; As, 18.46.

N-(Phenyl-4-arsonic Acid)glycyl-2-amino-benzamide, *p*-$H_2O_3AsC_6H_4NHCH_2CONHC_6H_4CONH_2$(*o*-).—After boiling equivalent amounts of sodium arsanilate, *o*-chloroacetylamino-benzamide,[1] and sodium iodide for two hours in 50% alcohol a clear solution resulted. Crystallization of the reaction product was started by rubbing with a rod and the crude product purified by dissolving in dil. sodium hydroxide and adding acetic acid. In this way it crystallizes slowly as faintly yellow, radiating masses of delicate microscopic needles which contain one molecule of water of crystallization. When rapidly heated to 165° and then slowly it sinters and slowly melts down at 170°. It is sparingly soluble in cold water, alcohol, or 50% alcohol, but readily soluble on warming. It is appreciably soluble in methyl alcohol in the cold.

Subs., air-dry, 0.8645: Loss, 0.0384 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{15}H_{16}O_5N_3As.H_2O$: H_2O, 4.38. Found: 4.44.
Subs., anhydrous, 0.1828: (Kjeldahl), 14.05 cc. 0.1 *N* HCl.
Calc. for $C_{15}H_{16}O_5N_3As$: N, 10.69. Found: 10.76.

Sodium Salt.—The neutral solution of the acid in a small volume of dil. sodium hydroxide, was treated with several volumes of alcohol.

[1] This Journal, 39, 1442 (1917).

On standing in the refrigerator the salt gradually crystallized. On recrystallization from 85% alcohol it formed globules of minute crystals which, like the acid, had a slightly yellow color. It contains 4.5 molecules of water of crystallization and dissolves readily in water.

Subs., air-dry, 0.5076: Loss, 0.0808 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{15}H_{15}O_5N_3AsNa.4.5H_2O$: H_2O, 16.33. Found: 15.92.
Subs., anhydrous, 0.3235: (Kjeldahl), 23.65 cc. 0.1 *N* HCl. Subs., 0.3825: $Mg_2As_2O_7$, 0.1441.
Calc. for $C_{15}H_{15}O_5N_3AsNa$: N, 10.13; As, 18.06. Found: N, 10.24; As, 18.18.

N-(Phenyl-4-arsonic Acid)glycyl-3-amino-benzamide.—As in the previous case *m*-chloroacetylamino-benzamide[1] yielded after several hours' boiling a supersaturated solution of the reaction product which crystallized only on chilling and rubbing. On treating a solution of the crude product in dil. sodium hydroxide with acetic acid the pure arsonic acid separated as irregular, microscopic platelets. Recrystallized from hot 50% alcohol, in which it is rather sparingly soluble, it separates as rosets of microscopic spears which contain between 2 and 2.5 molecules of water of crystallization. The acid is practically insoluble in boiling water, and when rapidly heated, darkens and shows signs of decomposition above 200° but does not decompose completely until 280° is reached.

Subs., air-dry, 0.9688: Loss, 0.0891 *in vacuo* at 100° over P_2O_5.
Calc. for $C_{15}H_{16}O_5N_3As.2H_2O$: H_2O, 8.39; 2.5 H_2O, 10.27. Found: 9.20.
Subs., anhydrous, 0.2713: (Kjeldahl), 20.5 cc. 0.1 *N* HCl; $Mg_2As_2O_7$, 0.1075.
Calc. for $C_{15}H_{16}O_5N_3As$: N, 10.69; As, 19.07. Found: N, 10.59; As, 19.13.

N-(Phenyl-4-arsonic Acid)glycyl-3-aminobenzoyl-urea, *p*-$H_2O_3AsC_6H_4NHCH_2CONHC_6H_4CONHCONH_2$(*m*-).—In this case the sparingly soluble *m*-chloroacetyl-aminobenzoyl-urea,[2] was gradually replaced by the new arsonic acid. After 4 hours[2] heating the resulting product was collected, suspended in water, and treated with dil. sodium carbonate solution until the solution remained slightly alkaline on thorough mixing and the arsonic acid was completely extracted from an insoluble residue. This was collected in the cold with boneblack and the clear filtrate treated with sodium acetate until the sodium salt

[1] This Journal, **39**, 1442 (1917).
[2] *Ibid.*, **39**, 2430 (1917).

separated. After standing in the refrigerator the salt was filtered off and washed with 20% sodium acetate solution, then with 85% alcohol. For purification it was dissolved in a small volume of water and treated with two volumes of alcohol. On scratching the sodium salt separated as thick, colorless masses of delicate microscopic hairs which contained 8 molecules of water of crystallization.

Subs., air-dry, 0.5560: Loss, 0.1323 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{16}H_{16}O_6N_4AsNa.8H_2O$: H_2O, 23.93. Found: 23.80.
Subs., anhydrous, 0.1335: 14.45 cc. N (26.0°, 756 mm.). Subs., 0.2892: $Mg_2As_2O_7$, 0.0970.
Calc. for $C_{16}H_{16}O_6N_4AsNa$: N, 12.23; As, 16.36. Found: N, 12.29; As, 16.19.

On addition of acetic acid to the hot dilute solution of the salt the free *arsonic acid* separates as sheaves and plumes of microscopic needles which decompose at about 280° with preliminary darkening and sintering. It is practically insoluble in boiling water or 50% alcohol.

Subs., 0.1663: (Kjeldahl), 15.25 cc. 0.1 *N* HCl.
Calc. for $C_{16}H_{17}O_6N_4As$: N, 12.84. Found: 12.84.

N-(Phenyl-4-arsonic Acid)glycyl-4-amino-benzamide.—The reaction in the case of *p*-chloroacetylamino-benzamide[1] occurred quickly as evidenced by the rapid disappearance of the sparingly soluble chloroacetyl compound, followed soon after by the sudden precipitation of the new arsonic acid. This was suspended in water and dissolved by the careful addition of dil. sodium hydroxide solution. After neutralization to litmus with acetic acid the solution was heated, filtered, and the filtrate treated with sodium acetate until the sodium salt began to separate. This was recrystallized from hot water, in which it is easily soluble, separating on cooling as rosets of thin, microscopic needles which contain 4.5 molecules of water of crystallization.

Subs., air-dry, 0.3410: Loss, 0.0564 *in vacuo* at 100° over P_2O_5.
Calc. for $C_{15}H_{15}O_5N_3AsNa.4.5H_2O$: H_2O, 16.33. Found: 16.54.
Subs., anhydrous, 0.3187: (Kjeldahl), 22.5 cc. 0.1 *N* HCl; $Mg_2As_2O_7$, 0.1210.
Calc. for $C_{15}H_{15}O_5N_3AsNa$: N, 10.13; As, 18.06. Found: N, 9.89; As, 18.33.

A hot, dilute solution of the salt, treated with acetic acid, immediately deposited the insoluble *arsonic acid* as nodules of microscopic needles which do not melt below 280°.

[1] This Journal, **39**, 1443 (1917).

Subs., 0.1034: 9.7 cc. N (22.0°, 757 mm.).
Calc. for $C_{15}H_{16}O_5N_3As$: N, 10.69. Found: 10.81.

N-(Phenyl-4-arsonic Acid)glycyl-5-amino-salicylamide, *p*-$H_2O_3AsC_6H_4NHCH_2CONHC_6H_3(OH)CONH_2$(*p*,*m*-).—42 g. of 5-chloroacetylamino-salicylamide,[1] by the sodium iodide method, after 2 hours boiling and purification of the crude product in the usual way, yielded 45 g. of the pure arsonic acid as glistening scales which contained one molecule of water of crystallization. When rapidly heated the anhydrous substance softens above 190° and gradually decomposes until fluid at about 255°. It is very sparingly soluble in boiling water or 50% alcohol, such solutions giving a brownish purple color with ferric chloride. In alkaline solution it couples with diazotized sulfanilic acid.

Subs., air-dry, 0.7932: Loss, 0.0356 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{15}H_{16}O_6N_3As.H_2O$: H_2O, 4.40. Found: 4.49.
Subs., anhydrous, 0.3148 (Kjeldahl), 22.5 cc. 0.1 *N* HCl; $Mg_2As_2O_7$, 0.1179.
Calc. for $C_{15}H_{16}O_6N_3As$: N, 10.27; As, 18.32. Found: N, 10.01; As, 18.08.

N-(Phenyl-4-arsonic Acid)-glycyl-3-amino-phenylacetamide, *p*-$H_2O_3AsC_6H_4NHCH_2CONHC_6H_4CH_2CONH_2$(*m*-).—4.6 g. of *m*-chloroacetylamino-phenylacetamide,[2] using sodium iodide, gave a clear solution from which the crude product separated on dilution and rubbing. The substance was redissolved in dil. ammonia and treated with acetic acid, the pure arsonic acid separating as masses of microscopic needles which darken slightly above 220° and decompose at 275–80°. It is soluble in boiling water and 50% alcohol. The yield was 5.2 g.

Subs., 0.1427: 12.8 cc. N (22.0°, 764 mm.). Subs., 0.3065: $Mg_2As_2O_7$, 0.1166.
Calc. for $C_{16}H_{18}O_5N_3As$: N, 10.32; As, 18.41. Found: N, 10.44; As, 18.35.

N-(Phenyl-4-arsonic Acid)glycyl-4-amino-phenylacetic Acid, *p*-$H_2O_3AsC_6H_4NHCH_2CONHC_6H_4CH_2CO_2H$(*p*-).—15.4 g. of arsanilic acid were dissolved in 70 cc. of *N* sodium hydroxide solution and boiled for one hour with 16 g. of *p*-chloroacetylamino-phenylacetic acid.[3]

[1] This Journal, **39**, 2420 (1917).
[2] *Ibid.*, **39**, 2421 (1917).
[3] *Ibid.*, **41**, 469 (1919).

The oil which separated on cooling soon crystallized and was triturated with 70 cc. of *N* hydrochloric acid solution, filtered off, washed with water, and dried. The yield of crude product, 24 g., was dissolved in dil. sodium hydroxide solution, made faintly acid with acetic acid, treated with boneblack, and the filtrate diluted to about 500 cc., warmed on the water bath, and treated with hydrochloric acid until just acid to congo red. The arsonic acid which separated was recrystallized from 85% alcohol, separating slowly as practically colorless, microscopic globules. The dried acid gradually darkens on heating, finally melting and decomposing on keeping the bath at 280° for a few moments. The anhydrous substance is pale yellow, losing its color without dissolving when boiled with water. It is quite soluble in boiling 85% alcohol or methyl alcohol and dissolves only sparingly in boiling acetic acid.

Subs., 0.2077: (Kjeldahl), 10.25 cc. 0.1 *N* HCl. Subs., 0.2747: $Mg_2As_2O_7$, 0.1038.
Calc. for $C_{16}H_{17}O_6N_2As$: N, 6.87; As, 18.36. Found: N, 6.91; As, 18.24.

N-(Phenyl-4-arsonic Acid) glycyl-4-amino-phenylacetamide.—(From *p*-chloroacetylamino-phenylacetamide[1] by the 50% alcohol and sodium iodide method.) The crude acid was reprecipitated from its solution in hot dil. ammonia by acetic acid, separating as plumes of microscopic hairs. The arsonic acid is only sparingly soluble in boiling water or 50% alcohol and does not melt below 280°. Because of the aliphatic amide linking it is very sensitive to fixed alkali.

Subs., 0.1305: 11.6 cc. N (24.0°, 771 mm.). Subs., 0.3092: $Mg_2As_2O_7$, 0.1192.
Calc. for $C_{16}H_{18}O_5N_3As$: N, 10.32; As, 18.41. Found: N, 10.3; As, 18.58.

Sodium Salt.—The acid was suspended in a small volume of water and very cautiously treated with dil. sodium hydroxide solution until just dissolved. The solution was then carefully neutralized to litmus with acetic acid and alcohol added until crystallization began. The salt separated as glistening platelets which, after air-drying, contained 2.5 molecules of water of crystallization and dissolved readily in water.

Subs., air-dry, 0.3945: Loss, 0.0371 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{16}H_{17}O_5N_3AsNa.2.5H_2O$: H_2O, 9.49. Found: 9.40.
Subs., anhydrous, 0.1489: 12.8 cc. N (22.0°, 756 mm.). Subs., 0.2625: $Mg_2As_2O_7$, 0.0936.
Calc. for $C_{16}H_{17}O_5N_3AsNa$: N, 9.79; As, 17.47. Found: N, 9.91; As, 17.21.

[1] This Journal, **39**, 1444 (1917).

N-(Phenyl-4-arsonic Acid)glycyl-4-amino-phenylacetureide, *p*-$H_2O_3AsC_6H_4NHCH_2CONHC_6H_4CH_2CONHCONH_2$(*p*-).—The very sparingly soluble *p*-chloroacetylamino-phenylacetylurea,[1] was very slowly and incompletely replaced by the reaction product. After 5 hours' boiling the somewhat gelatinous mass was diluted with water and filtered, and in order to extract the desired arsonic acid completely the residue was placed in a bottle with several hundred cc. of water containing a slight excess of ammonia and the mixture shaken in a machine until thoroughly disintegrated. Boneblack was then added and the mixture again shaken in order to collect the insoluble, gelatinous material. The filtrate was heated and acidified with acetic acid, causing the separation of the crystalline arsonic acid. This was converted into the *sodium salt* by suspending in a small volume of warm water and very cautiously treating with dil. sodium hydroxide until solution was just complete and the reaction was only faintly alkaline to litmus. The filtered solution was warmed and treated with alcohol until turbid, the salt separating on cooling as microscopic, hexagonal plates which contained 3 molecules of water of crystallization. The yield was 4.7 g.

Subs., air-dry, 0.4511: Loss, 0.0483 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{17}H_{18}O_6N_4AsNa.3H_2O$: H_2O, 10.27. Found: 10.71.
Subs., anhydrous, 0.1500: 15.6 cc. N (25.0°, 758 mm.). Subs., 0.2521: $Mg_2As_2O_7$, 0.0815.
Calc. for $C_{17}H_{18}O_6N_4AsNa$: N, 11.87; As, 15.88. Found: N, 11.88; As, 15.61.

On adding acetic acid to a hot solution of the sodium salt the free *arsonic acid* separated gradually as plumes of minute hairs which are almost insoluble in boiling water or 50% alcohol. It darkens slightly, but does not melt below 280°.

Subs., 0.1538: (Kjeldahl), 13.45 cc. 0.1 *N* HCl.
Calc. for $C_{17}H_{19}O_6N_4As$: N, 12.45. Found: 12.25.

N-(Phenyl-4-arsonic Acid)glycyl-2-amino-phenoxyacetamide, *p*-$H_2O_3AsC_6H_4NHCH_2CONHC_6H_4OCH_2CONH_2$(*o*-).—The reaction product from *o*-chloroacetylamino-phenoxyacetamide,[2] was dissolved in dil. ammonia in order to avoid danger of saponification of the amide

[1] This Journal, **39**, 2434 (1917).
[2] *Ibid.*, **39**, 2422 (1917).

group. The addition of acetic acid to the heated filtrate caused the separation of the acid as a voluminous mass of microscopic needles which contained one molecule of water of crystallization. The yield was 10 g. The acid is very sparingly soluble in boiling water and somewhat more so in hot 50% alcohol, from which it crystallizes in minute, glistening needles on cooling. When anhydrous it decomposes at about 280° with preliminary darkening.

Subs., air-dry, 0.0845: Loss, 0.0033 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{16}H_{18}O_6N_3As.H_2O$: H_2O, 4.08. Found: 3.90.
Subs., anhydrous, 0.1370: 11.95 cc. N (23.0°, 756 mm.). Subs., 0.3369: $Mg_2As_2O_7$, 0.1222.
Calc. for $C_{16}H_{18}O_6N_3As$: N, 9.93; As, 17.72. Found: N, 10.02; As, 17.50.

N-(Phenyl-4-arsonic Acid)glycyl-3-amino-phenoxyacetic Acid, *p*-$H_2O_3AsC_6H_4NHCH_2CONHC_6H_4OCH_2CO_2H$(*m*-).—In this case the addition of sodium iodide as a catalyzer was unnecessary. 22 g. of arsanilic acid were dissolved in 100 cc. of *N* sodium hydroxide solution, 25 g. of *m*-chloroacetylamino-phenoxyacetic acid[1] were added and the mixture boiled for one hour. An oil separated from the clear solution on cooling. On the addition of hydrochloric acid in excess the new arsonic acid rapidly crystallized. After filtration it was dissolved in sufficient dil. sodium hydroxide solution, diluted to about 500 cc. and warmed. On adding hydrochloric acid until congo red paper turned color, a finely divided, oily suspension was formed which solidified on rubbing, forming radiating masses of microcrystals containing approximately one molecule of water of crystallization. The yield was 28 g. The acid is fairly easily soluble in boiling water or 50% alcohol. When rapidly heated the anhydrous substance softens at 180–90°, then darkens and finally decomposes at about 250–60°.

Subs., air-dry, 0.5668: Loss, 0.0197 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{16}H_{17}O_7N_2As.H_2O$: H_2O, 4.07. Found: 3.47.
Subs., anhydrous, 0.1583: (Kjeldahl), 7.53 cc. 0.1 *N* HCl. Subs., 0.3019. $Mg_2As_2O_7$, 0.1088.
Calc. for $C_{16}H_{17}O_7N_2As$: N, 6.60; As, 17.68. Found: N, 6.66; As, 17.40.

N-(Phenyl-4-arsonic Acid)glycyl-4-amino-phenoxyacetic Acid.—This substance was obtained as in the case of the previously described *meta*

[1] This Journal, **41**, 466 (1919).

isomer, from *p*-chloroacetylamino-phenoxyacetic acid.[1] After 30 minutes' boiling the clear solution suddenly set to a thick, crystalline mass, after which heating was continued 30 minutes longer on the water bath. The product was dissolved in sufficient sodium hydroxide solution, diluted to about 500 cc., heated on the water bath, and acidified to congo red with hydrochloric acid. The arsonic acid immediately separated as a colorless powder, which appeared as minute globules under the microscope. The yield was 30 g. When a hot, dilute solution of the salt (see below) is acidified with acetic acid the arsonic acid gradually separates as aggregates of flat, microscopic needles and platelets. It is practically insoluble in hot alcohol, methyl alcohol, or water, but is appreciably soluble in boiling 50% alcohol. The acid darkens above 250° but does not melt below 285°.

Subs., 0.1495: (Kjeldahl), 7.05 cc. 0.1 *N* HCl.
Calc. for $C_{16}H_{17}O_7N_2As$: N, 6.61. Found: 6.59.

Sodium Salt.—On addition of alcohol to a neutral solution of the acid in dil. sodium hydroxide solution until crystallization started, the sodium salt separated as glistening, microscopic leaflets which contained 3 molecules of water of crystallization.

Subs., air-dry, 0.5258: Loss, 0.0535 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{16}H_{15}O_7N_2AsNa_2.3H_2O$: H_2O, 10.34. Found: 10.18.
Subs., anhydrous, 0.1441: 7.5 cc. N (23.0°, 765 mm.). Subs., 0.3417: $Mg_2As_2O_7$, 0.1133.
Calc. for $C_{16}H_{15}O_7N_2AsNa_2$: N, 5.99; As, 16.02. Found: N, 6.05; As, 16.02.

N-(Phenyl-4-arsonic Acid)glycyl-4-amino-phenoxyacetamide.—16 g. of *p*-chloroacetylamino-phenoxyacetamide[2] were employed, using sodium iodide. The reaction product which separated during the heating was purified by dissolving in dil. ammonia and reprecipitating with acetic acid. The yield was 16.5 g. The acid separates from hot, dilute solutions of its salts on adding acetic acid as sheaves and plumes of minute, flat needles which do not melt below 280°. It is very sparingly soluble in boiling water or 50% alcohol.

Subs., 0.1433: 12.6 cc. N (22.0°, 747 mm.).
Calc. for $C_{16}H_{15}O_6N_3As$: N, 9.93. Found: 10.01.

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[1] This Journal, 39, 2436 (1917).
[2] *Ibid.*, 39, 2423 (1917).

Sodium Salt.—As the amide group is rather sensitive to fixed alkali, sodium carbonate was used to dissolve the acid suspended in a small volume of hot water. The sodium salt began to separate on cooling and was more completely precipitated by adding sodium acetate solution. Recrystallized from water it formed long, flat, microscopic needles containing 5 molecules of water of crystallization.

Subs., air-dry, 1.3832: Loss, 0.2362 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{16}H_{17}O_6N_3AsNa.5H_2O$: H_2O, 16.83. Found: 17.08.
Subs., anhydrous, 0.2846: (Kjeldahl), 19.3 cc. 0.1 *N* HCl. Subs., 0.2936: $Mg_2As_2O_7$, 0.1010.
Calc. for $C_{16}H_{17}O_6N_3AsNa$: N, 9.44; As, 16.84. Found: N, 9.50; As, 16.60.

N-(Phenyl-4-arsonic Acid)glycyl-4-amino-phenoxyacetylurea, *p*-$H_2O_3AsC_6H_4NHCH_2CONHC_6H_4OCH_2CONHCONH_2$(*p*-).—5.8 g. of the almost insoluble *p*-chloroacetylamino-phenoxyacetylurea[1] were gradually replaced by the new arsonic acid. After 4 hours the filtered product was suspended in warm water and sodium carbonate added until the arsonic acid was completely extracted from an insoluble residue and the solution remained just alkaline to litmus. The use of sodium hydroxide was avoided owing to the lability of the ureide group. The insoluble residue was collected with bone-black and the clear filtrate salted out with sodium acetate. After standing in the refrigerator the filtered salt was washed with 20% sodium acetate solution and then 85% alcohol, and purified by dissolving in a small volume of warm water and treating with two volumes of alcohol. On cooling the *sodium salt* gradually separated as aggregates of microscopic needles containing 4 molecules of water of crystallization. The yield was 4.5 g.

Subs., air-dry, 0.3254: Loss, 0.0419 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{17}H_{18}O_7N_4AsNa.4H_2O$: H_2O, 12.86. Found: 12.88.
Subs., anhydrous, 0.1301: 13.0 cc. N (25.0°, 766 mm.). Subs., 0.1479: $Mg_2As_2O_7$, 0.0467.
Calc. for $C_{17}H_{18}O_7N_4AsNa$: N, 11.48; As, 15.36. Found: N, 11.54; As, 15.24.

On adding acetic acid to a hot, dilute solution of the salt the *arsonic acid* separated as rosets of microscopic needles which contained ½ mole-

[1] This Journal, **39**, 2435, 2436 (1917).

cule of water of crystallization. The anhydrous acid slowly decomposes at about 290° with preliminary darkening, and is very difficultly soluble in boiling water or 50% alcohol.

Subs., air-dry, 0.4852: Loss, 0.0071 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{17}H_{19}O_7N_4As.0.5H_2O$: H_2O, 1.89. Found: 1.46.
Subs., anhydrous, 0.1580: (Kjeldahl), 13.45 cc. 0.1 *N* HCl.
Calc. for $C_{17}H_{19}O_7N_4As$: N, 12.02. Found: 11.92.

N-(Phenyl-4-arsonic Acid) glycyl-3-methyl-4-amino-phenoxyacetic Acid, p-$H_2O_3AsC_6H_4NHCH_2CONHC_6H_3(CH_3)OCH_2CO_2H(o,p-)$.—4.4 g. of arsanilic acid, dissolved in 20 cc. of *N* sodium hydroxide solution, and 5.2 g. of 3-methyl-4-chloroacetylamino-phenoxyacetic acid,[1] yielded a clear solution on boiling. On chilling after one hour an oil separated and slowly crystallized. A solution of the crude substance in dil. sodium hydroxide was warmed and acidified with hydrochloric acid to congo red, after which the small amount of flocculent precipitate carrying coloring matter was rapidly filtered off. On cooling and standing 3 g. of the arsonic acid slowly separated as a crust on the sides of the vessel. For analysis it was recrystallized from a small volume of 50% alcohol. As so obtained the substance decomposes from 250–60° with preliminary sintering. The acid is difficult to obtain in well-defined crystals as it easily forms supersaturated solutions, crystallizes very slowly, and separates as a gum when rapidly thrown out of solution. When a dilute solution of the sodium salt described below is acidified with acetic acid the substance separates very gradually as warty aggregates of microscopic needles which decompose at 270° with preliminary darkening. It is appreciably soluble in methyl alcohol and in boiling water and readily in hot methyl or ethyl alcohol.

Subs., 0.2008: (Kjeldahl), 9.08 cc. 0.1 *N* HCl. Subs., 0.1802: $Mg_2As_2O_7$, 0.0660.
Calc. for $C_{17}H_{19}O_7N_2As$: N, 6.39; As, 17.12. Found: N, 6.33; As, 17.68.

Sodium Salt.—When a hot, neutral solution of the acid in a small volume of sodium hydroxide solution was treated with alcohol it yielded an oil which gradually crystallized on cooling and standing, while well-defined needles slowly separated from the supernatant

[1] This Journal, **39**, 2201 (1917).

liquor. The salt obtained in this way contained water of crystallization but the amount was not determined. It is easily soluble in water.

Subs., anhydrous, 0.3198: (Kjeldahl), 13.45 cc. 0.1 *N* HCl; $Mg_2As_2O_7$, 0.1040.
Calc. for $C_{17}H_{18}O_7N_2AsNa_2$: N, 5.81; As, 15.54. Found: N, 5.89; As, 15.69.

N-(Phenyl-4-arsonic Acid)glycyl-3-amino-benzenesulfonamide, *p*-$H_2O_3AsC_6H_4NHCH_2CONHC_6H_4SO_2NH_2$ (*m*-).—Equivalent amounts of sodium arsanilate, *m*-chloroacetylamino-benzenesulfonamide,[1] and sodium iodide in 50% alcohol yielded a clear solution after several hours' boiling, from which the reaction product crystallized on cooling and rubbing. The collected product, after solution in dil. ammonia, was precipitated by acetic acid as flat, glistening, microscopic needles, often grouped in rosets. 21.5 g. of the chloroacetyl compound yielded 25 g. of the arsonic acid. It decomposes at about 265° with preliminary darkening and is difficultly soluble in boiling water or 50% alcohol.

Subs., 0.1755: 15 cc. N (23.0°, 756 mm.).
Calc. for $C_{14}H_{16}O_6N_3SAs$: N, 9.79. Found: 9.81.

N-(Phenyl-4-arsonic Acid)glycyl-4-amino-benzenesulfonic Acid, *p*-$H_2O_3AsC_6H_4NHCH_2CONHC_6H_4SO_3H$(*p*-).—16.5 g. of arsanilic acid were dissolved in 75 cc. of *N* sodium hydroxide solution and 25 g. of sodium chloroacetyl-sulfanilate[2] were added. After boiling the mixture for 30 minutes and cooling, the resulting clear solution was treated with an excess of hydrochloric acid. The arsonic acid separated on rubbing and was filtered off and washed with 10% hydrochloric acid. Reprecipitated from solution in dil. ammonia with hydrochloric acid, the pure arsonic acid separated slowly as flat, lustrous needles. The yield was 20 g. The acid is appreciably soluble in cold water, but less so in 10% hydrochloric acid solution. It is readily soluble in hot water, from which it separates on cooling as aggregates of minute, flat needles which contain two molecules of water of crystallization. It is sparingly soluble in alcohol and practically insoluble in acetone. The anhydrous substance slowly softens and decomposes at 245–6°.

[1] This Journal, **39**, 2429 (1917).
[2] *Ibid.*, **41**, 470 (1919).

Subs., air-dry, 0.8815: Loss, 0.0683 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{14}H_{15}O_7N_2SAs.2H_2O$: H_2O, 7.73. Found: 7.75.
Subs., anhydrous, 0.1537: (Kjeldahl), 7.14 cc. 0.1 *N* HCl. Subs., 0.3036: $Mg_2As_2O_7$, 0.1076.
Calc. for $C_{14}H_{15}O_7N_2SAs$: N, 6.51; As, 17.42. Found: N, 6.50; As, 17.11.

N-(Phenyl-4-arsonic Acid)glycyl-4-amino-benzenesulfonamide.—The product from 21.5 g. of *p*-chloroacetylamino-benzenesulfonamide[1] in the presence of sodium iodide, was dissolved in dil. ammonia. On acidification of the hot, dilute solution with acetic acid the new arsonic acid rapidly separated as colorless aggregates of thin, microscopic leaflets and needles. The yield was 30 g. The acid is insoluble in boiling water or 50% alcohol, and on rapid heating sinters slightly, but does not melt below 280°.

Subs., 0.1374: 11.6 cc. N (20.0°, 769 mm.). Subs., 0.3019: $Mg_2As_2O_7$, 0.1078.
Calc. for $C_{14}H_{16}O_6N_3SAs$: N. 9.79; As, 17.45. Found: N, 9.95; As, 17.23.

N-(Phenyl-4-arsonic Acid)glycyl-4-amino-6-hydroxybenzenesulfonic Acid, *p*-$H_2O_3AsC_6H_4NHCH_2CONHC_6H_3(OH)SO_3H$(*m,p*-).—22 g. of arsanilic acid in 100 cc. of *N* sodium hydroxide solution and 30 g. of the sodium salt of 4-chloroacetylamino-6-hydroxybenzenesulfonic acid[2] were boiled for 30 minutes and the clear solution then chilled and treated with 25 cc. of conc. hydrochloric acid. On scratching and standing in the refrigerator the reaction product crystallized and was filtered off and washed with 10% hydrochloric acid. The crude substance was suspended in water, treated with sodium acetate solution until completely dissolved, warmed, and the solution then treated with conc. hydrochloric acid until a concentration of about 10% hydrochloric acid was reached. On cooling the arsonic acid slowly separated as a thick crust of crystals which was washed with 10% hydrochloric acid and then with ice water. The yield was 25 g. The acid separates with approximately 1.5 molecules of water of crystallization and is fairly readily soluble in water at ordinary temperatures, but like many sulfonic acids it is less soluble in dil. hydrochloric acid. It separates from hot water on thorough chilling as microscopic leaflets and is difficultly soluble in hot alcohol, methyl alcohol, or acetic

[1] This Journal, **39**, 2429 (1917).
[2] *Ibid.*, **41**, 471 (1919).

acid. In alkaline solution it couples readily with diazotized sulfanilic acid. The anhydrous substance softens and darkens above 200°, but does not melt below 275°.

Subs., air-dry, 0.7833: Loss, 0.488 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{14}H_{15}O_8N_2SAs.1.5H_2O$: H_2O, 5.71. Found: 6.23.
Subs., anhydrous, 0.2022: (Kjeldahl), 9.25 cc. 0.1 *N* HCl. Subs., 0.2258: $Mg_2As_2O_7$, 0.0798.
Calc. for $C_{14}H_{15}O_8N_2SAs$: N, 6.28; As, 16.81. Found: N, 6.40; As, 17.06.

N-(Phenyl-4-arsonic Acid)glycyl-4-amino-acetophenone, *p*-$H_2O_3AsC_6H_4NHCH_2CONHC_6H_4COCH_3$(*p*-).—4.3 g. of *p*-chloroacetylamino-acetophenone[1] with sodium iodide yielded 4 g. of the crystalline arsonic acid, after purification in the usual manner. The acid separates from hot, dilute solutions of its salts on addition of acetic acid as long, fine hairs which do not melt below 280°. It is almost insoluble in boiling water and but sparingly so in hot 50% alcohol. It dissolves in conc. sulfuric acid with a yellow color.

Subs., 0.1538: 9.6 cc. N (25.5°, 768 mm.). Subs., 0.3164: $Mg_2As_2O_7$, 0.1240.
Calc. for $C_{16}H_{17}O_5N_2As$: N, 7.15; As, 19.10. Found: N, 7.22; As, 18.91.

(*B*) *Derivatives of o-Arsanilic Acid.*

N-(Phenyl-2-arsonic Acid)glycineanilide, *o*-$H_2O_3AsC_6H_4NHCH_2CONHC_6H_5$.—This substance was prepared in the same way as the *p*-derivative from *o*-arsanilic acid[2] and chloroacetanilide. The arsonic acid separated slowly on standing, and precipitation was completed by diluting the reaction mixture. The crude product was ground up with dil. hydrochloric acid and recrystallized from 50% alcohol, crystallizing on seeding as radiating masses of minute prisms with one molecule of water of crystallization. The yield equaled the amount of *o*-arsanilic acid used. The anhydrous substance softens at 158°, melts at 160–3° with slow gas evolution, and dissolves very easily in boiling 50% alcohol, although sparingly in the cold. It is very difficultly soluble in boiling water and also in cold acetic acid, but dissolves readily in the latter on warming. It is also easily soluble in cold methyl alcohol, less readily in ethyl alcohol.

[1] This Journal, **41**, 469 (1919).
[2] *Ibid.*, **40**, 1583 (1918).

Subs., air-dry, 0.6579: Loss, 0.0363 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{14}H_{15}O_4N_2As.H_2O$: H_2O, 4.89. Found: 5.52.
Subs., anhydrous, 0.2171: (Kjeldahl), 12.25 cc. 0.1 *N* HCl. Subs., 0.3047: $Mg_2As_2O_7$, 0.1364.
Calc. for $C_{14}H_{15}O_4N_2As$: N, 8.00; As, 21.39. Found: N, 7.91; As, 21.60.

N-(Phenyl-2-arsonic Acid)glycyl-2-aminophenol.—22 g. of *o*-arsanilic acid and 19 g. of *o*-chloroacetylamino-phenol in 200 cc. of 0.5 *N* sodium hydroxide solution were boiled for $\frac{1}{2}$ hour. On cooling the clear solution deposited an oil, part of which dissolved on rendering the mixture ammoniacal, leaving a copious residue of *o*-amino-phenoxyacetic anhydride.[1] On acidifying the filtrate with hydrochloric acid the oil which first separated was soon followed by crystals, and on standing in the ice box the entire product crystallized. The crude substance was dissolved in dil. ammonia and the solution then acidified faintly with acetic acid and treated with boneblack. On adding hydrochloric acid carefully to the hot filtrate until just acid to congo red the arsonic acid separated as beautiful balls of glistening needles which soon filled the liquid. The yield was 8 g., containing 0.5 molecule of water of crystallization. When anhydrous it melts and decomposes at 151–3°. From hot water, in which it is but sparingly soluble, it separates on cooling as long, silky needles. It is fairly readily soluble in cold alcohol or methyl alcohol and easily so in hot 50% alcohol. An alkaline solution couples readily with diazotized sulfanilic acid.

Subs., air-dry, 0.7450: Loss, 0.0197 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{14}H_{15}O_5N_2As.0.5H_2O$: H_2O, 2.40. Found: 2.64.
Subs., anhydrous, 0.2520: (Kjeldahl), 14.0 cc. 0.1 *N* HCl. Subs., 0.2734: $Mg_2As_2O_7$, 0.1178.
Calc. for $C_{14}H_{15}O_5N_2As$: N, 7.66; As, 20.48. Found: N, 7.78; As, 20.79.

N-(Phenyl-2-arsonic Acid)glycyl-3-aminophenol.—22 g. of *o*-arsanilic acid dissolved in 100 cc. of *N* sodium hydroxide solution and 19 g. of *m*-chloroacetylamino-phenol[2] were boiled for 30 minutes. On cooling a deep red oil separated, but on adding hydrochloric acid until acid to congo red, it crystallized on rubbing. The substance was then

[1] Cf. the *p*-arsonic acids, pp. 199, 200.
[2] This Journal, **39**, 1442 (1917).

filtered off, washed with water, suspended in a small volume of water, and dissolved by the addition of ammonia. On rendering slightly acid with acetic acid the deep colored solution deposited a dark, gummy precipitate which was collected in the cold with boneblack and filtered. The filtrate was then heated with boneblack, which removed most of the color. A pink color still persisted in the filtrate, a property which all of the substances derived from *m*-chloroacetyl-amino-phenol have shown. The filtrate was diluted to about 500 cc., heated, and then treated with hydrochloric acid until congo red turned color, causing the momentary separation of an oil which on stirring rapidly crystallized, forming bundles of pink microscopic platelets which when air-dried contained approximately two molecules of water of crystallization and melted at 103–5°. The yield was 17 g. When anhydrous the substance softens at about 125–30° and melts completely with decomposition at about 180°. It is readily soluble in alcohol, methyl alcohol, acetic acid, and hot water, and in alkaline solution couples readily with diazotized sulfanilic acid.

Subs., air-dry, 0.6155: Loss, 0.0590 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{14}H_{15}O_5N_2As.2H_2O$: H_2O, 8.96. Found: 9.59.
Subs., anhydrous, 0.2355: (Kjeldahl), 12.55 cc. 0.1 *N* HCl. Subs., 0.3123: $Mg_2As_2O_7$, 0.1362.
Calc. for $C_{14}H_{15}O_5N_2As$: N, 7.66; As, 20.48. Found: N, 7.46; As, 21.04.

N-(Phenyl-2-arsonic Acid)glycyl-4-aminophenol. — Equimolecular quantities of *o*-arsanilic acid and *p*-chloroacetylamino-phenol yielded a dark colored solution from which an oil separated on cooling. This slowly solidified, especially after making definitely acid to congo red with hydrochloric acid. After standing in the ice box the product was filtered off, washed, dissolved in dil. ammonia, and the solution faintly acidified with acetic acid and treated with boneblack. On acidifying the hot filtrate to congo red with hydrochloric acid the arsonic acid again separated as an oil which soon crystallized. This substance was again dissolved in dil. ammonia and the solution acidified with acetic acid and again treated with boneblack to remove the color which still persisted. On diluting the filtrate to about 300 cc., heating, and adding hydrochloric acid until congo red paper just turned and as long as the oily emulsion which momentarily formed

redissolved, the arsonic acid separated on scratching as a heavy powder, consisting of short, colorless, microscopic platelets. The filtered substance was washed with water. The yield was 16 g. from 22 g. of *o*-arsanilic acid. When rapidly heated the acid darkens and then melts and decomposes at 208–9°. It is very sparingly soluble in boiling water and easily in boiling methyl alcohol or 50% alcohol.

Subs., 0.2331: (Kjeldahl), 12.9 cc. 0.1 *N* HCl. Subs., 0.2950: $Mg_2As_2O_7$, 0.1255.
Calc. for $C_{14}H_{15}O_5N_2As$: N, 7.66; As, 20.48. Found: N, 7.75; As, 20.53.

(*C*) *Derivatives of m-Arsanilic Acid.*

N-(Phenyl-3-arsonic Acid)glycineanilide.—(From *m*-arsanilic acid[1] and chloroacetanilide.) On diluting the reaction mixture with water and rendering acid to congo red with hydrochloric acid the crystalline arsonic acid was gradually deposited. This was recrystallized first from 50% alcohol and finally from glacial acetic acid, separating from the latter as rosets of minute, cream-colored prisms which apparently contained solvent of crystallization. When rapidly heated to 215° and then slowly the dried substance darkens, then softens, and finally decomposes at 217–18°. It is rather sparingly soluble in hot water, but dissolves readily in hot 50% alcohol or acetic acid and is appreciably soluble in cold 95% alcohol.

Subs., 0.2422: (Kjeldahl), 14.25 cc. 0.1 *N* HCl. Subs., 0.2857: $Mg_2As_2O_7$, 0.1267.
Calc. for $C_{14}H_{15}O_4N_2As$: N, 8.00; As, 21.39. Found: N, 8.24; As, 21.40.

N-(Phenyl-3-arsonic Acid)glycyl-2-aminophenol.—Although the reaction between *m*-arsanilic acid and *o*-chloroacetylamino-phenol was accompanied by the formation of *o*-amino-phenoxyacetic anhydride, as in the case of *p*- and *o*-arsanilic acids, the amount of anhydride was much less than that formed when the isomeric acids were used. Consequently the yield of the complex arsonic acid was much greater than in the case of the *o*- or *p*-arsanilic acid.

The reaction mixture from 22 g. of *m*-arsanilic acid and 19 g. of *o*-chloroacetylamino-phenol yielded an oil on cooling which redissolved on rendering the mixture ammoniacal, leaving about 3 g. of *o*-amino-phenoxyacetic anhydride. The filtrate was acidified to congo red

[1] This Journal, **40**, 1583 (1918).

with hydrochloric acid, causing the deposition of a viscous oil which slowly crystallized on rubbing and letting stand. After standing for 24 hours in the refrigerator the crude substance was filtered off and washed with water. The deep colored solution of this product in dil. ammonia was neutralized with acetic acid and treated with bone-black. The still colored filtrate was diluted to about 300 cc., heated on the water bath, and then treated with hydrochloric acid until congo red turned color. On scratching and keeping sufficiently warm the substance slowly crystallized as almost colorless, flat, microscopic needles. If allowed to chill it separated first as an oil carrying coloring matter with it. If properly heated and with constant manipulation with a rod crystallization was almost complete in about 15 minutes.

After cooling it was filtered off and washed with water. The yield was 21 g. When rapidly heated to 185°, then slowly, the arsonic acid darkens and softens, finally melting and decomposing at 190–2°. It is sparingly soluble in cold water, more easily on boiling, and on cooling it again separates very slowly and incompletely. It is somewhat soluble in the cold in 50% or 95% alcohol, more easily on warming, and also dissolves in cold methyl alcohol or boiling acetic acid. A solution of the acid in strong ammonium acetate solution slowly deposits the ammonium salt as minute needles. Although hydrochloric acid precipitates the acid fairly completely from its salts as a gummy mass, an excess of acetic acid causes no immediate precipitate. However, on long standing the free arsonic acid separates slowly and incompletely from such solutions as a crust of broad, microscopic needles.

Subs., 0.1835: (Kjeldahl), 10.35 cc. 0.1 *N* HCl. Subs., 0.3004: $Mg_2As_2O_7$, 0.1282.

Calc. for $C_{14}H_{15}O_5N_2As$: N, 7.66; As, 20.48. Found: N, 7.90; As, 20.57.

N-(Phenyl-3-arsonic Acid)glycyl-3-aminophenol.—26.4 g. of *m*-arsanilic acid and 23 g. of *m*-chloroacetylamino-phenol yielded a purple oil which slowly crystallized on scratching. Attempts at purifying this substance by the usual method of reprecipitating the acid from its solution in dil. alkali by either acetic or hydrochloric acid were unsuccessful since the compound easily formed supersaturated solutions from which it separated extremely slowly, usually as highly colored,

amorphous flocks. The substance was best purified by dissolving in hot 50% acetic acid and treating with boneblack, most of the purple color still persisting in the filtrate. On standing in the refrigerator the acid gradually separated as a thick crust of crystals which carried down the color with them. The yield was 27.5 g. A further recrystallization was then attempted from 50% alcohol, the solution depositing the substance slowly as a light purple powder consisting of minute, irregular platelets and flat needles containing approximately 1.5 molecules of water of crystallization. When rapidly heated to 150° the anhydrous substance gradually sinters to a tar which decomposes at about 180–90°. In the cold it is more soluble in methyl alcohol than in the other solvents, but is fairly easily soluble in hot water, more so in hot alcohol, acetic acid, or in these solvents when diluted. It is practically insoluble in acetone, benzene, or ether.

Subs., air-dry, 0.3468: Loss, 0.0210 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{14}H_{15}O_5N_2As.1.5H_2O$: H_2O, 6.87. Found: 6.06.
Subs., anhydrous, 0.1607: (Kjeldahl), 8.95 cc. 0.1 *N* HCl. Subs., 0.2531: $Mg_2As_2O_7$, 0.1058.
Calc. for $C_{14}H_{15}O_5N_2As$: N, 7.66; As, 20.48. Found: N, 7.80; As, 20.18.

N-(Phenyl-3-arsonic Acid)glycyl-4-aminophenol.—The almost colorless oil obtained from 22 g. of *m*-arsanilic acid and 19 g. of *p*-chloroacetylamino-phenol could not be made to crystallize directly and was therefore dissolved by adding an equal volume of conc. hydrochloric acid to the reaction mixture, with chilling. On scratching, the clear solution deposited the hydrochloride of the arsonic acid as minute needles, the mixture setting to a thick mass after several hours. After filtering off, the hydrochloride was washed with 1:1 hydrochloric acid and was then suspended in water and dissolved by the addition of ammonia. The warm solution was treated with hydrochloric acid until acid to congo red, causing the partial separation of the substance as an oil which gradually crystallized on scratching. After standing in the refrigerator it was filtered off and washed with ice water. The yield was 21 g.. When recrystallized from a small volume of hot water, in which it readily dissolves, it separates on rapid chilling as a milky emulsion which then very slowly crystallizes, partly as a hydrate. But if allowed to cool very slowly it separates gradually as

microscopic platelets which are anhydrous. When rapidly heated the arsonic acid starts to sinter and darken above 170° and when held at 180° slowly shrinks together and decomposes. It is sparingly soluble in cold water but dissolves readily in the boiling solvent. It is easily soluble in hot alcohol, appreciably so in cold methyl alcohol, and but sparingly soluble in hot acetone. It dissolves in warm, dil. hydrochloric acid and on addition of the stronger acid it is converted into the hydrochloride, which separates slowly on rubbing.

Subs., 0.1527: (Kjeldahl), 8.47 cc. 0.1 *N* HCl. Subs., 0.3042: $Mg_2As_2O_7$, 0.1308. Calc. for $C_{14}H_{15}O_5N_2As$: N, 7.66; As, 20.48. Found: N, 7.77; As, 20.75.

(*D*) *Derivatives of Substituted p-Arsanilic Acids.*

N-(2-Methylphenyl-4-arsonic Acid)glycyl-3-aminophenol.—30 g. of the sodium salt of *o*-methylarsanilic acid (from *o*-toluidine) and 17 g. of *m*-chloroacetylamino-phenol, yielded an oil which rapidly crystallized on acidifying the mixture to congo red with hydrochloric acid. After dissolving the product in dil. ammonia and acidifying the solution with acetic acid, 17 g. of the pure arsonic acid separated as long, flat, microscopic needles. When heated to 285° the acid darkens and slowly decomposes. It is practically insoluble in boiling water, but is appreciably dissolved by hot 50% alcohol. An alkaline solution couples readily with diazotized sulfanilic acid.

Subs., 0.1338: 8.6 cc. N (28.5°, 763 mm.). Subs., 0.2924: $Mg_2As_2O_7$, 0.1182. Calc. for $C_{15}H_{17}O_5N_2As$: N, 7.37; As, 19.72. Found: N, 7.31; As, 19.50.

N-(2-Methylphenyl-4-arsonic Acid)glycyl-4-aminophenol.—This substance was obtained exactly as in the case of its isomer and in about the same yield. On acidifying a hot solution of the arsonic acid in dil. ammonia with acetic acid the free acid separates as microscopic, spindle-shaped needles. When rapidly heated it decomposes at 232–3° with preliminary darkening. It is very sparingly soluble in boiling water and slightly more soluble in hot 50% alcohol.

Subs., 0.1313: 8.6 cc. N (28.0°, 761 mm.). Subs., 0.3246: $Mg_2As_2O_7$, 0.1347. Calc. for $C_{15}H_{17}O_5N_2As$: N, 7.37; As, 19.72. Found: N, 7.44; As, 20.01.

N-(3-Methylphenyl-4-arsonic Acid)glycyl-3-aminophenol.—This substance was obtained as in the case of the two previously described

compounds, starting with 2-methyl-4-aminophenyl arsonic acid.[1] Acetic acid slowly precipitates the compound from hot ammoniacal solution as aggregates of spindle-shaped microcrystals. When rapidly heated it decomposes at 232–5° with preliminary softening and darkening. It is very sparingly soluble in boiling water or alcohol, more readily in hot 50% alcohol. An alkaline solution couples readily with diazotized sulfanilic acid.

Subs., 0.1682: 10.6 cc. N (21.0°, 755 mm.). Subs., 0.3100: $Mg_2As_2O_7$, 0.1275.
Calc. for $C_{15}H_{17}O_5N_2As$: N, 7.37; As, 19.72. Found: N, 7.28; As, 19.84.

N-(2-Carboxyphenyl-4-arsonic Acid)glycyl-3-aminophenol, *o,p*-$HO_2C(H_2O_3As)C_6H_3NHCH_2CONHC_6H_4OH$(*m*-).—10 g. of 3-carboxy-4-aminophenyl arsonic acid,[2] were dissolved in 80 cc. of *N* sodium hydroxide solution (about 2 mols.), then treated with 20 cc. of 2 *N* acetic acid and 8 g. of *m*-chloroacetylamino-phenol, and the mixture boiled for one hour. The reaction was apparently facilitated by the presence of the sodium acetate as buffer, since without the addition of acetic acid the solution would have reacted alkaline and decomposed the chloroacetylamino-phenol. On acidifying the clear, chilled solution with hydrochloric acid the reaction product crystallized. This was purified by redissolving in dil. sodium hydroxide, rendering faintly acid with acetic acid and adding boneblack in the cold to collect a small amount of flocculent material. The filtrate was warmed and then treated with an excess of acetic acid, the arsonic acid separating on scratching as thin, minute platelets. After washing with water and air-drying, the yield was 6 g. The acid separates with one molecule of water of crystallization and is practically insoluble in boiling water. It is somewhat soluble in hot alcohol or 50% alcohol and dissolves fairly readily in boiling methyl alcohol or glacial acetic acid. When rapidly heated the anhydrous substance darkens and swells, then decomposes at 204–7°. An alkaline solution couples readily with diazotized sulfanilic acid.

Subs., air-dry, 0.5776: Loss, 0.0236 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{15}H_{15}O_7N_2As.H_2O$: H_2O, 4.21. Found: 4.09.
Subs., anhydrous, 0.1441: (Kjeldahl), 7.1 cc. 0.1 *N* HCl. Subs., 0.3292: $Mg_2As_2O_7$, 0.1231.
Calc. for $C_{15}H_{15}O_7N_2As$: N, 6.83; As, 18.28. Found: N, 6.90; As, 18.03.

[1] This Journal, **40**, 1588 (1918).
[2] Kahn and Benda, *Ber.*, **41**, 3862 (1908).

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AROMATIC ARSENIC COMPOUNDS. V.

N-Substituted Glycylarsanilic Acids.

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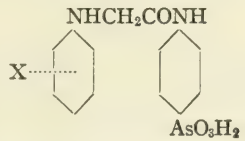
The substances discussed in the present communication are closely related to the previously described substituted anilides of phenylglycine-*p*-arsonic acid,[1] but differ from these in the fact that the glycine side chain is reversed, the arsonic acid radical becoming a substituent on the anilide nucleus, as follows:



These substances were readily prepared by boiling the sodium salt of chloroacetyl-arsanilic acid with the aromatic amino compound, the condensation occurring in boiling aqueous solution within 15 to 30 minutes, owing to the reactivity of the chlorine in the former substance. The first member of the series, glycylarsanilic acid, was prepared by the use of ammonia in this reaction and was accompanied by the formation of the secondary imino compound. With the aromatic amines no difficulty was experienced in any case and the reaction has proved capable of wide extension. We have confined our studies up to the present to those compounds which were prepared for biological investigation.

The fundamental compound required in this work was chloroacetylarsanilic acid. This derivative has already been mentioned in German patent 191,548, but as far as we could ascertain it has not been employed for the synthesis of other arsenic compounds. The patent contains a method for its preparation by the interaction of arsanilic

[1] This Journal, **41**, 1610 (1919).

acid and chloroacetyl chloride, but we have found it unsuitable for the preparation of the substance in large quantities as the reaction was difficult to control and the yields were poor and uncertain. A very serviceable and simple means was found for the large scale preparation of chloroacetyl-arsanilic acid, consisting in heating arsanilic acid with an excess of chloroacetic acid on the water bath, an excellent yield of the pure substance being easily obtained.

The substituted phenylglycyl-arsanilic acids resemble in general properties the isomeric anilides of phenylglycine-*p*-arsonic acid, functioning both as acids and feeble bases. They are but sparingly soluble in the usual solvents and all have high melting or decomposition points, the observed values depending upon the rate of heating. In general, the sodium salts were readily soluble in water.

EXPERIMENTAL.

Chloroacetyl-arsanilic Acid, *p*-$H_2O_3AsC_6H_4NHCOCH_2Cl$.—A method for the preparation of chloroacetyl-arsanilic acid has been given in German patent 191,548. In addition to the fact that no full description of the substance is given, we have found the following method more serviceable for its preparation in large quantity: 50 g. of arsanilic acid and 150 g. of dry chloroacetic acid are heated in an open flask on the water bath for 2 hours. The mixture gradually forms a clear melt, and towards the end the chloroacetyl-arsanilic acid occasionally begins to separate. The melt is poured into about a liter of well stirred, saturated sodium chloride solution, and the precipitated arsonic acid is filtered off and washed well with water. Yield, 50 g. A portion of the acid was dissolved in hot water, treated with bone black, and the filtered solution cooled rapidly in order to prevent decomposition. Under these conditions it separates as minute, lenticular platelets and toothed leaflets which contain no water of crystallization and which darken when rapidly heated, but melt and decompose only when the temperature of the bath is held at 285° for a few moments. The acid is readily soluble in hot alcohol, somewhat less so in boiling water or acetic acid, and sparingly soluble in the last two in the cold and in acetone. It is slowly acted upon by prolonged exposure to moist air.

Subs., 0.1867; (Kjeldahl) 6.25 cc. 0.1 *N* HCl. Subs., 0.1342; (Carius), AgCl, 0.0634.
Subs., 0.2824; $Mg_2As_2O_7$, 0.1481.
Calc. for $C_8H_9O_4NClAs$: N, 4.77; Cl, 12.08; As, 25.53. Found: N, 4.69; Cl, 11.69; As, 25.31.

Practically as good an analysis was obtained on the crude product, and this was used in all of the following preparations:

Glycyl-p-arsanilic Acid, *p*-$H_2NCH_2CONHC_6H_4AsO_3H_2$.—20 g. of chloroacetyl-arsanilic acid were added to 100 cc. of conc. aqueous ammonia, warmed gently to about 30° until solution was complete, and allowed to stand overnight. The excess of ammonia was then boiled off from the clear solution, causing the crude reaction product to crystallize. After cooling this was filtered off, 12 g. being obtained. The product was purified by treating with dil. hydrochloric acid, avoiding a large excess on account of the consequent precipitation of the hydrochloride of the glycine. After filtering off the imino compound (see below) the crude glycylaminophenyl-arsonic acid was precipitated by adding sodium acetate. For further purification it was dissolved in just enough dil. sodium hydroxide solution and then precipitated by means of a stream of carbon dioxide. A repetition of the final purification was necessary before good analytical figures could be obtained, a considerable loss of material also taking place. The pure amino acid forms minute, toothed, glistening plates which are anhydrous, do not melt below 295°, and are very difficultly soluble in boiling water or 50% alcohol. It functions as a very weak acid, being soluble only in an excess of weak bases, such as ammonia, and is displaced from its salts by carbon dioxide.

Subs., 0.1328; 11.8 cc. N (24.5°, 751 mm.). Subs., 0.3101; $Mg_2As_2O_7$, 0.1743.
Calc. for $C_8H_{11}O_4N_2As$: N, 10.22; As, 27.34. Found: N, 10.08; As, 27.12.

Iminobisacetyl-p-arsanilic Acid, $(p\text{-}H_2O_3AsC_6H_4NHCOCH_2)_2NH$.—The hydrochloric acid-insoluble fraction obtained in the preparation of the *p*-glycylarsanilic acid was dissolved in dil. sodium hydroxide solution, precipitated with an excess of hydrochloric acid, further purified by a repetition of this process, and finally recrystallized by dissolving in hot, dil. ammonium hydroxide and adding acetic acid in excess. The imino acid separated as glistening rosets of microcrystals.

Before filtering, the solution was cooled and acidified to congo red with hydrochloric acid. The substance darkens at 280–5° without melting, and is practically insoluble in the usual solvents.

Subs., 0.1318; 9.2 cc. N (29.0°, 757 mm.). Subs., 0.3401; $Mg_2As_2O_7$, 0.1987.
Calc. for $C_{16}H_{19}O_8N_3As_2$: N, 7.92; As, 28.23. Found: N, 7.86; As, 28.19.

N-Methylglycyl-p-arsanilic Acid, *p*-$CH_3NHCH_2CONHC_6H_4AsO_3H_2$.—A strong methylamine solution was prepared by treating 25 g. of methylamine hydrochloride with 60 g. of well chilled, 25% sodium hydroxide solution. Without removing the precipitated sodium chloride, 5 g. of chloroacetyl-arsanilic acid were added. On allowing to warm to room temperature and breaking up the lumps with a rod, the acid soon dissolved. After 24 hours' standing the excess of methylamine was removed on the water bath and on diluting the residue and acidifying strongly with acetic acid the arsonic acid slowly crystallized. Recrystallized from water it separates as long, silky, glistening needles which contain approximately two molecules of water of crystallization. The yield was 2 g. It turns brown at about 250° but does not melt below 275°. It is practically insoluble in hot alcohol and is not very freely soluble in boiling water. It dissolves in dilute mineral acids and on adding sodium nitrite to the solution in dil. hydrochloric acid the nitroso derivative deposits on rubbing as sheaves and spheres of microscopic needles.

Subs., air-dry, 0.5641; loss, 0.0670 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_9H_{13}O_4N_2As.2H_2O$: H_2O, 11.12. Found: 11.87.
Subs., anhydrous, 0.1864; (Kjeldahl) 12.85 cc. 0.1 *N* HCl. Subs., 0.2635; $Mg_2As_2O_7$, 0.1404.
Calc. for $C_9H_{13}O_4N_2As$: N, 9.73; As, 26.00. Found; N, 9.66; As, 25.72.

N-Phenylglycyl-p-arsanilic Acid (*N-Phenylglycineanilide-p'-arsonic Acid*), *p*-$C_6H_5NHCH_2CONHC_6H_4AsO_3H_2$.—This substance, isomeric with phenylglycineanilide-*p*-arsonic acid,[1] was prepared as follows: 2 g. of aniline in 15 cc. of alcohol were added to a solution of 4.5 g. of chloroacetyl-arsanilic acid in 15 cc. of *N* sodium hydroxide solution. The solution was boiled for ½ hour under an air-condenser, finally setting to a crystalline cake. The yield of acid was 4.4 g. Recrystallized

[1] This Journal, **41**, 1613 (1919).

from 50% alcohol the arsonic acid forms delicate, felted needles which are almost insoluble in boiling water. When heated it darkens slightly, but does not melt below 280°. When a suspension of the acid in hot 50% acetic acid is treated with excess sodium nitrite it dissolves at once, the nitroso compound soon crystallizing from the solution as nacreous plates.

Subs., 0.3260; (Kjeldahl) 18.70 cc. 0.1 *N* HCl; $Mg_2As_2O_7$, 0.1435.
Calc. for $C_{14}H_{15}O_4N_2As$: N, 8.00; As, 21.39. Found: N, 8.04; As, 21.24.

m-Oxaminophenyl-glycyl-p-arsanilic Acid, *m*-$HO_2CCONHC_6H_4NH$-$CH_2CONHC_6H_4AsO_3H_2(p')$.—12 g. of chloroacetyl-arsanilic acid and 7.2 g. of *m*-amino-oxanilic acid[1] were dissolved in 80 cc .of *N* sodium hydroxide solution and boiled for 20 minutes. The small amount of crystalline material which separated on cooling was filtered off and the filtrate made acid to congo red. A dark-colored gum separated, but the supernatant liquid, on standing overnight, deposited a crystalline crust. This was dissolved in very dilute aqueous ammonia and hydrochloric acid added to the solution until a faint turbidity persisted. This contained much coloring matter and was filtered off. It was found that if hydrochloric acid was added directly to the filtrate only a caseous precipitate could be obtained, but if acetic acid was first added in sufficient amount and then hydrochloric acid until definitely blue to congo red paper, the solution, when seeded, deposited the *hydrochloride* of the oxamino acid as aggregates of microscopic plumes, which gradually lost hydrochloric acid on standing in moist air.

Subs., 0.1546; 12.0 cc. N (17.5°, 750 mm.).
Calc. for $C_{16}H_{16}O_7N_3As.HCl$: N, 8.88. Found: 9.00.

A portion of the hydrochloride was boiled with water, causing a change in physical appearance and the elimination of hydrochloric acid. The free *oxamino acid* was filtered off, washed well with hot water, and air-dried, one molecule of water of crystallization being retained. The substance is practically insoluble in boiling water and very sparingly so in 50% alcohol. When rapidly heated to 175°, then slowly, the anhydrous acid effervesces at 179°, with preliminary softening and darkening.

[1] This Journal, **39**, 1451 (1917).

Subs., air-dry, 0.5870; loss, 0.0249 at 100° *in vacuo* over H_2SO_4.
Calc. for $C_{16}H_{16}O_7N_3As.H_2O$: H_2O, 3.96. Found: H_2O, 4.24.
Subs., anhydrous, 0.1412; 11.85 cc. N (23.5°, 757 mm.). Subs., 0.3966, $Mg_2As_2O_7$, 0.1389.
Calc. for $C_{16}H_{16}O_7N_3As$: N, 9.61; As, 17.13. Found: N, 9.62; As, 16.90.

p-Aminophenyl-glycyl-p-arsanilic Acid, p-$H_2NC_6H_4NHCH_2CONH$-$C_6H_4AsO_3H_2$.—8 g. of *p*-acetamino-phenylglycineanilide-*p'*-arsonic acid (see below) were boiled with 8 parts of 1:1 hydrochloric acid for 6 minutes, cooled rapidly, shaken with bone black, filtered, and treated with saturated sodium acetate solution until the disappearance of the congo red reaction. The precipitate was filtered off, dissolved in dil. hydrochloric acid, and the solution filtered and treated again with sodium acetate. The amino acid separated as microcrystalline aggregates which tended to become colloidal on washing with water. The yield was 4.5 g. When rapidly heated the acid darkens and sinters, but does not melt below 280°. It is soluble in dilute mineral acids and alkalies and is practically insoluble in boiling water and very sparingly so in boiling 50% alcohol. It is readily diazotized, giving a red color with R-salt.

Subs., 0.1425; 14.15 cc. N (21.5°, 751 mm.). Subs., 0.3254; $Mg_2As_2O_7$, 0.1367.
Calc. for $C_{14}H_{16}O_4N_3As$: N, 11.51; As, 20.52. Found: N, 11.38; As, 20.27.

p-Acetaminophenyl-glycyl-p-arsanilic Acid.—Starting with 6 g. of *p*-aminoacetanilide, the reaction product began to separate after a few minutes, the heating being continued for one hour on the water bath. The yield of crude acid was 12 g. This was converted into the *sodium salt* by dissolving in warm, dil. sodium hydroxide solution, and salting out the neutral solution with saturated sodium acetate solution. Recrystallized from water containing a small amount of sodium carbonate to prevent hydrolysis to the free acid, it separated as flat, glistening needles which contain 7 molecules of water of crystallization when air-dry. It may also be precipitated from its aqueous solution by the addition of alcohol.

Subs., air-dry, 0.4734; loss, 0.1057 at 100° *in vacuo* over H_2SO_4.
Calc. for $7H_2O$: H_2O, 22.72. Found: 22.32.
Subs., anhydrous, 0.2998; (Kjeldahl) 20.50 cc. 0.1 *N* HCl. Subs., 0.3346; $Mg_2As_2O_7$, 0.1193.
Calc. for $C_{16}H_{17}O_5N_3AsNa$: N, 9.79; As, 17.45. Found: N, 9.58; As, 17.22.

On treating a hot dilute solution of the sodium salt with acetic acid, the free *arsonic acid* separated slowly as glistening, hexagonal platelets. It does not melt below 275° and is very sparingly soluble in boiling water or 50% alcohol.

Subs., 0.2117; (Kjeldahl) 15.4 cc. 0.1 *N* HCl.
Calc. for $C_{16}H_{18}O_5N_3As$: N, 10.32. Found: 10.19.

p-Oxaminophenyl-glycyl-p-arsanilic Acid.—14 g. of chloroacetyl-arsanilic acid and 8.4 g. of *p*-amino-oxanilic acid[1] were boiled with 94 cc. of *N* sodium hydroxide solution for ½ hour, the product separating after about 10 minutes. The yield was 16.5 g. Reprecipitated from very dilute, hot ammoniacal solution with acetic acid, the arsonic acid separates as slightly purplish aggregates of microscopic crystals containing 1.5 molecules of water of crystallization. It is almost insoluble in boiling water, very sparingly so in boiling 50% alcohol, and when anhydrous, darkens above 200°, but does not melt below 275.° On heating with aqueous alkali in attempts to hydrolyze the oxamino group the principal products recovered were either the unchanged oxamino compound or arsanilic acid, depending on the length of heating and the strength of alkali. Heating, however, with 1:1 hydrochloric acid yielded the amino compound.

Subs., air-dry, 0.4539; loss, 0.0281 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{16}H_{16}O_7N_3As.1.5H_2O$; H_2O, 5.82. Found: 6.19.
Subs., anhydrous, 0.1441; 12.2 cc. N (24.0°, 754 mm.). Subs., 0.4027; $Mg_2As_2O_7$, 0.1418.
Calc. for $C_{16}H_{16}O_7N_3As$: N, 9.61; As, 17.13. Found: N, 9.65; As, 16.99.

p-Oxamylaminophenyl-glycyl-p-arsanilic Acid, p-$H_2NCOCONHC_6H_4NHCH_2CONHC_6H_4AsO_3H_2$.—21 g. of chloroacetyl-arsanilic acid were dissolved in 70 cc. of *N* sodium hydroxide solution and added to a boiling solution of 12.6 g. of *p*-amino-oxanilamide[2] in about 300 cc. of water. On continuing the boiling the product separated as a partly gelatinous mass which gradually crystallized. After ½ hour the hot solution was filtered and the precipitate washed with hot water. It was then suspended in a large volume of hot water and treated with

[1] This Journal, 39, 1459 (1917).
[2] *Ibid.*, 39, 1461 (1917).

sodium carbonate solution until only a small, gelatinous residue remained. The mixture was treated with bone black and filtered through a hot-water funnel in order to prevent deposition of the sodium salt on the filter. The filtrate was warmed and treated with sodium acetate until crystallization of the salt commenced and was then let stand in the refrigerator. On treating a very dilute, hot solution of the salt with acetic acid, the free arsonic acid separated as clusters of microscopic needles in a yield of 6.5 g. It is practically insoluble in boiling water or 50% alcohol, and does not melt below 285°.

Subs., 0.1299; 14.65 cc. N (24.0°, 763 mm.). Subs., 0.2065; $Mg_2As_2O_7$, 0.0723. Calc. for $C_{16}H_{17}O_6N_4As$: N, 12.85; As, 17.19. Found: N, 13.02; As, 16.90.

p-Uraminophenyl-glycyl-p-arsanilic Acid, p-$H_2NCONHC_6H_4NHCH_2CONHC_6H_4AsO_3H_2$.—12 g. of chloroacetyl-arsanilic acid, 6.1 g. of *p*-aminophenylurea,[1] 40 cc. of *N* sodium hydroxide solution, and 20 cc. of saturated sodium acetate solution were boiled for ½ hour. On cooling a small amount of precipitate was filtered off and the solution then saturated with sodium acetate and let stand in the ice-box. After 24 hours the precipitate of sodium salt was filtered off, washed with 85% alcohol, dissolved in warm water, treated with bone black, filtered, and the acid precipitated from the filtrate by means of acetic acid. The yield was 7 g. The uramino acid separates as spherular masses of microscopic leaflets which dissolve in boiling 50% alcohol and very sparingly in water. When rapidly heated it changes color above 150° and darkens markedly at about 210°, but does not melt below 285°.

Subs., 0.1471; 17.7 cc. N (26.0°, 762 mm.). Subs., 0.1858; $Mg_2As_2O_7$, 0.0696. Calc. for $C_{15}H_{17}O_5N_4As$: N, 13.72; As, 18.35. Found: N, 13.78; As, 18.08.

m-Hydroxyphenyl-glycyl-p-arsanilic Acid, m-$HOC_6H_4NHCH_2CONHC_6H_4AsO_3H_2$.—Since the oily reaction product from 11 g. of *m*-aminophenol was difficult to obtain in crystalline form, purification was effected by conversion into the *hydrochloride* by adding 100 cc. of 1:1 hydrochloric acid to the reaction mixture. The salt was filtered off and washed with 10% hydrochloric acid. A portion, recrystallized

[1] This Journal, 39, 1456 (1917).

from 10% hydrochloric acid, separated as slightly purplish microcrystals which blacken above 140° but do not melt up to 280°. The hydrochloride loses most of its halogen on boiling with water.

Subs., 0.2152; (Kjeldahl) 10.6 cc. 0.1 *N* HCl.
Calc. for $C_{14}H_{15}O_5N_2As.HCl$: N, 6.96. Found: 6.90.

The main portion of this salt was suspended in water and dissolved by the addition of sodium hydroxide solution. On acidifying with hydrochloric acid until just faintly acid to congo red a considerable amount of gummy material was precipitated and was collected with bone black and quickly filtered off. The clear, almost colorless filtrate deposited the crystalline *arsonic acid* on scratching. It was purified by again dissolving in dil. sodium hydroxide solution and acidifying with an excess of acetic acid. The pure arsonic acid separated as pale pink, wedge-shaped, microscopic prisms which contained 3.5 molecules of water of crystallization after air-drying. The yield was 15.5 g. On rapid heating the arsonic acid melts at about 80° in its water of crystallization, but rapidly resolidifies. On further heating it turns purple at about 180° and then gradually softens and chars as the temperature is raised. It is easily soluble in ethyl or methyl alcohol or acetone. It dissolves less readily in glacial acetic acid and then separates on scratching in a different form, presumably owing to dehydration. Dilution with water redissolves the crystals. It dissolves readily in boiling water and separates on cooling as a caseous mass.

Subs., air-dry, 0.6257; loss, 0.0893 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{14}H_{15}O_5N_2As.3.5H_2O$: H_2O, 14.68. Found: 14.27.
Subs., anhydrous, 0.1682; (Kjeldahl) 9.35 cc. 0.01 *N* HCl. Subs., 0.2541; $Mg_2As_2O_7$, 0.1064.
Calc. for $C_{14}H_{15}O_5N_2As$: N, 7.66; As, 20.48. Found: N, 7.78; As, 20.21.

p-Hydroxyphenyl-glycyl-p-arsanilic Acid.—(From 4.5 g. of *p*-aminophenol.) After a few minutes the reaction product separated from the clear solution and the mixture was then transferred to the water bath and heated one hour. For purification the precipitate was dissolved by neutralization of an aqueous suspension with sodium hydroxide, and the filtered solution treated with 1:1 hydrochloric acid until the precipitate which first formed had redissolved. The copious

precipitate of the hydrochloride of the arsonic acid which separated on scratching was filtered off. Yield, 11 g. On dissolving in a small volume of dil. sodium hydroxide solution and exactly neutralizing with acetic acid, the *sodium salt* separated on standing in the ice-box. Recrystallized from a small volume of water, it forms aggregates of microscopic needles which contain one molecule of water of crystallization and dissolve sparingly in water at 0° and readily on warming. The aqueous solution of the salt gives a slowly developing pale lilac color with ferric chloride.

Subs., air-dry, 0.3783; loss, 0.0143 at 100° *in vacuo* over H_2SO_4.
Calc. for $C_{14}H_{14}O_5N_2AsNa.H_2O$: H_2O, 4.43. Found: 3.78.
Subs., anhydrous, 0.2833; (Kjeldahl) 14.0 cc. 0.1 *N* HCl; $Mg_2As_2O_7$, 0.1134.
Calc. for $C_{14}H_{14}O_5N_2AsNa$: N, 7.22; As, 19.30. Found: N, 6.92; As, 19.32.

On acidifying a hot solution of the salt with acetic acid the free *arsonic acid* separates as aggregates of microscopic pairs which contain approximately one molecule of water of crystallization. The substance is almost insoluble in boiling water or 50% alcohol, and, when anhydrous, blackens and sinters above 200°, but does not melt entirely below 280°.

Subs., air-dry, 0.4903; loss, 0.0265 at 100° *in vacuo* over H_2SO_4.
Calc. for $C_{14}H_{15}O_5N_2As.H_2O$: H_2O, 4.69. Found: 5.40.
Subs., anhydrous, 0.1532; 10.2 cc. N (21.5°, 750 mm.).
Calc. for $C_{14}H_{15}O_5N_2As$: N, 7.65. Found: 7.62.

m-Carboxamidophenyl-glycyl-p-arsanilic Acid, *m*-$H_2NCOC_6H_4NHCH_2CONHC_6H_4AsO_3H_2$(*p*).—After one hour 5.5 g. of *m*-amino-benzamide[1] yielded an oil on cooling which readily crystallized, giving 10.5 g. of the crude arsonic acid. The neutral solution of the crude acid in dil. sodium hydroxide was salted out with several volumes of saturated sodium acetate solution, and the *sodium salt* recrystallized by treating its warm concentrated solution with alcohol until a slight permanent turbidity remained. Under these conditions the salt crystallized as radiating masses of flat needles containing one molecule of water of crystallization.

[1] This Journal, 39, 1438 (1917).

Subs., air-dry, 0.6223; loss, 0.0276 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{15}H_{15}O_5N_3AsNa.H_2O$: H_2O, 4.16. Found: 4.44.
Subs., anhydrous, 0.2967; (Kjeldahl) 21.3 cc. 0.1 *N* HCl; $Mg_2As_2O_7$, 0.1098.
Calc. for $C_{15}H_{15}O_5N_3AsNa$: N, 10.13; As, 18.06. Found: N, 10.06; As, 17.87.

On treating a hot solution of the sodium salt with acetic acid, the *arsonic acid* gradually separated on scratching as microscopic needles. It is very difficultly soluble in boiling water or acetic acid but dissolves somewhat more easily in boiling 50% alcohol. When rapidly heated to 245°, then slowly, it darkens and then decomposes at 248°.

Subs., 0.2137; (Kjeldahl) 23.0 cc. 0.1 *N* HCl.
Calc. for $C_{15}H_{16}O_5N_3As$: N, 10.68. Found: 10.76.

N-Phenylglycineanilide-m-carboxureide-p'-arsonic Acid, *p*-H_2O_3As-$C_6H_4NHCOCH_2NHC_6H_4CONHCONH_2$(*m*).—Starting with 5.4 g. of *m*-aminobenzoylurea[1] a clear solution was rapidly obtained and the arsonic acid was deposited during the heating. After cooling and filtering the acid was suspended in a little water, treated with a very slight excess of sodium hydroxide and filtered from a little insoluble material after exactly neutralizing with acetic acid. The addition of powdered, crystalline sodium acetate to the filtrate yielded the *sodium salt*. This was dissolved in a small volume of hot water and treated with an equal volume of alcohol. On standing in the cold the salt separated as flat, microscopic needles containing 3 molecules of water of crystallization. The yield was 7.3 g.

Subs., air-dry, 0.5948; loss, 0.0625 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{16}H_{16}O_6N_4AsNa.3H_2O$: H_2O, 10.55. Found: 10.50.
Subs., anhydrous, 0.1443; 15.35 cc. N (23.0°, 756 mm.). Subs., 0.3214; $Mg_2As_2O_7$, 0.1073.
Calc. for $C_{16}H_{16}O_6N_4AsNa$: N, 12.23; As, 16.36. Found: N, 12.21; As, 16.11.

On adding acetic acid to a dilute solution of the salt the *free acid* separated slowly as spherules of microscopic needles which darken when rapidly heated and decompose at about 280°. The acid is almost insoluble in boiling water or 50% alcohol.

Subs., 0.1441; 15.8 cc. N (20.0°, 758 mm.).
Calc. for $C_{16}H_{17}O_6N_4As$: N, 12.84. Found: 12.74.

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[1] This Journal, 39, 1439 (1917).

p-Carboxamidophenyl-glycyl-p-arsanilic Acid, *p*-$H_2NCOC_6H_4NH$-$CH_2CONHC_6H_4AsO_3H_2(p)$.—This substance, prepared from 5.5 g. of *p*-aminobenzamide,[1] separated from the boiling reaction mixture within a few moments, the heating being continued on the water bath for one hour longer and the arsonic acid then filtered off, washed with water and alcohol, and dried. The yield was 13 g. It was suspended in about 100 cc. of warm water, dissolved by neutralizing with sodium hydroxide, and the solution filtered and let stand in the ice-box. 13 g. of the *sodium salt* separated as glistening platelets containing 2 molecules of water of crystallization. The salt is quite sparingly soluble in ice water, more easily on warming.

Subs., air-dry, 1.0357; loss, 0.0857 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{15}H_{15}O_5N_3AsNa.2H_2O$: H_2O, 7.99. Found: 8.27.
Subs., anhydrous, 0.2995; (Kjeldahl) 21.3 cc. 0.1 *N* HCl; $Mg_2As_2O_7$, 0.1135.
Calc. for $C_{15}H_{15}O_5N_3AsNa$: N, 10.13; As, 18.06. Found: N, 9.96; As, 18.29.

Addition of acetic acid to the filtrate from the sodium salt gave a small amount of the *free acid* as microscopic prisms which do not melt below 275° and are practically insoluble in boiling water or 50% alcohol.

Subs., 0.2075; (Kjeldahl) 15.6 cc. 0.1 *N* HCl.
Calc. for $C_{15}H_{16}O_5N_3As$: N, 10.69. Found 10.53.

N-Phenylglycineanilide-p-acetamide-p'-arsonic Acid, H_2O_3As-C_6H_4-$NHCOCH_2NH$-C_6H_4-CH_2CONH_2. — (From *p*-aminophenyl-acetamide.[2]) The oil which separated on cooling soon crystallized on rubbing, and after dilution of the mixture with water, the crude substance was filtered off, washed, and dissolved in dilute aqueous ammonia. On adding a slight excess of acetic acid an amorphous impurity quickly separated. This was filtered off, after which the pure acid separated slowly as aggregates of microscopic platelets which darken and soften above 180° and decompose completely at 256–8°. It dissolves with difficulty in boiling water, more easily in boiling 50% alcohol or acetic acid.

[1] This Journal, **39**, 1436 (1917), paragraph 2.
[2] *Ibid.*, **39**, 1444 (1917).

Subs., 0.1360; 12.0 cc. N (25.5°, 761 mm.). Subs., 0.3214; $Mg_2As_2O_7$, 0.1210.
Calc. for $C_{16}H_{18}O_5N_3As$: N, 10.32; As, 18.41. Found: N, 10.11; As. 18.17.

N-Phenylglycineanilide-p-acetureide-p'-arsonic Acid, $H_2O_3AsC_6H_4NHCOCH_2NHC_6H_4CH_2CONHCONH_2$.—(From *p*-amino-phenylacetylurea.[1]) The clear solution which immediately formed on boiling set after a few minutes to a solid mass of crystals and it was necessary to continue the heating on the water bath. After diluting with water, the crude product was filtered off, washed, taken up in a little hot water, and treated with sodium carbonate until faintly alkaline. The insoluble, gelatinous residue was collected with bone black and the filtrate treated with sodium acetate until the sodium salt of the arsonic acid began to separate. The salt was converted into the free acid by addition of acetic acid to its hot, dilute solution. The product so obtained was further purified by reprecipitating its very dilute, hot, ammoniacal solution with acetic acid. The pure arsonic acid separated slowly as rosets of microscopic hairs which contained 0.5 molecule of water of crystallization. The anhydrous compound darkens above 230° and decomposes at 270–3°. It is sparingly soluble in boiling water or 50% alcohol.

Subs., air-dry, 0.5047; loss, 0.0094 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{17}H_{19}O_6N_4As.0.5H_2O$: H_2O, 1.96. Found: 1.86.
Subs., anhydrous, 0.1409; 15.3 cc. N (22.0°, 756 mm.). Subs., 0.2920; $Mg_2As_2O_7$, 0.1017.
Calc. for $C_{17}H_{19}O_6N_4As$: N, 12.45; As, 16.64. Found: N, 12.49; As, 16.81.

N-Phenylglycineanilide-p-hydroxyacetic Ether Acid-p'-arsonic Acid, $H_2O_3As\langle\rangle NHCOCH_2HN\langle\rangle OCH_2COOH$.—3 g. of chloroacetyl-arsanilic acid and 2 g. of *p*-aminophenoxyacetic acid[2] were dissolved in 20 cc. of *N* sodium hydroxide solution and boiled for ½ hour. The crude reaction product was filtered off and recrystallized from 50% acetic acid, separating as minute, pale brown, wedge-shaped plates containing 1.5 molecules of water of crystallization. The acid is almost insoluble in boiling water or 50% alcohol, and when anhydrous darkens above 180° and decomposes at about 275°.

[1] This Journal, **39**, 2433 (1917).
[2] *Ibid.*, **39**, 2196 (1917).

Subs., air-dry, 0.6434; loss, 0.0404 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{16}H_{17}O_7N_2As.1.5H_2O$: H_2O, 5.99. Found: 6.28.
Subs., anhydrous, 0.3070; (Kjeldahl) 14.9 cc. 0.1 *N* HCl. Subs., 0.3300; $Mg_2As_2O_7$, 0.1205.
Calc. for $C_{16}H_{17}O_7N_2As$: N, 6.61; As, 17.67. Found: N, 6.80; As, 17.62.

N-Phenylglycineanilide-p-hydroxyacetic Ether Acid Amide-p'-arsonic Acid, *p*-$H_2O_3AsC_6H_4NHCOCH_2NHC_6H_4OCH_2CONH_2(p)$.—(From 4 g. of *p*-aminophenoxyacetamide.[1]) The new arsonic acid separated almost at once and after heating on the water bath for an hour longer it was filtered off and washed with water. A suspension of the acid in a small volume of water was treated with a very slight excess of sodium hydroxide, quickly made exactly neutral to litmus with acetic acid, and the dark solution filtered. After adding sodium acetate to the filtrate the *sodium salt* separated on standing and was filtered off, dissolved in a little water, and reprecipitated with one to two volumes of alcohol. It crystallized in slightly grayish rosets of flat, glistening needles which, after washing with 85% alcohol and air-drying, contained 4 molecules of water of crystallization. The yield was 3.2 g. A dilute, aqueous solution of the salt gives immediate precipitates with silver or copper ions, a slow-forming, crystalline precipitate with Ca^{++}, and no precipitate with Ba^{++}.

Subs., air-dry, 0.5303; loss, 0.0720 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{16}H_{17}O_6N_3AsNa.4H_2O$: H_2O, 13.93. Found: 13.57.
Subs., anhydrous, 0.1465; 12.4 cc. N (24.0°, 750 mm.). Subs., 0.3069; $Mg_2As_2O_7$, 0.1055.
Calc. for $C_{16}H_{17}O_6N_3AsNa$; N, 9.44; As, 16.84. Found: N, 9.61; As, 16.59.

The *free acid* obtained from the salt with acetic acid separates slowly as woolly masses of delicate needles which are practically insoluble in boiling water or 50% alcohol and which darken somewhat but do not melt below 265°.

Subs., 0.1484; (Kjeldahl) 10.45 cc. 0.1 *N* HCl.
Calc. for $C_{16}H_{18}O_6N_3As$: N, 9.93. Found: 9.87.

N-Phenylglycineanilide-p-hydroxyacetic Ether Acid Ureide-p'-arsonic Acid, *p*-$H_2O_3AsC_6H_4NHCOCH_2NHC_6H_4OCH_2CONHCONH_2$.—Starting with 2.2 g. of *p*-amino-phenoxyacetylurea,[2] the new arsonic acid

[1] This Journal, **39**, 2423 (1917).
[2] *Ibid.*, **39**, 2435 (1917).

separated during the heating. The washed product was dissolved in hot, dil. sodium acetate solution, filtered, and saturated sodium acetate solution added to the filtrate until crystallization of the *sodium salt* started. For final purification this was dissolved in a little hot water and the solution treated with alcohol until crystallization again started. After letting stand in the cold the salt was filtered off, washed with 85% alcohol, and air-dried. As so obtained it forms rosets of minute leaflets which contain 4 molecules of water of crystallization.

Subs., air-dry, 0.4633; loss, 0.0595 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{17}H_{18}O_7N_4AsNa.4H_2O$: H_2O, 12.86. Found: 12.84.
Subs., anhydrous, 0.1575; 15.4 cc. N (22.0°, 764 mm.).
Calc. for $C_{17}H_{18}O_7N_4AsNa$: N, 11.48. Found: 11.38.

On acidification of a hot solution of the salt with acetic acid the *free acid* was obtained as feathery aggregates of minute platelets which are almost insoluble in boiling water or 50% alcohol. When rapidly heated to 255°, then slowly, the acid decomposes at 257–8° with preliminary darkening.

Subs., 0.1680; (Kjeldahl) 14.25 cc. 0.1 *N* HCl. Subs., 0.3140; $Mg_2As_2O_7$, 0.1034.
Calc. for $C_{17}H_{19}O_7N_4As$: N, 12.02; As, 16.07. Found: N, 11.88; As, 15.89.

N-Phenylglycineanilide-p-glycineamide-p'-arsonic Acid, $H_2O_3As\langle\bigcirc\rangle NHCOCH_2N(H)\langle\bigcirc\rangle NHCH_2CONH_2$. — Carefully purified *p*-aminophenyl-glycineamide[1] yielded a thick oil which slowly crystallized on rubbing and letting stand, and in subsequent preparations crystallization, could be immediately induced by seeding and continued warming on the water bath. The mixture was filtered hot in order to eliminate the tar which separated from the filtrate on cooling. The arsonic acid was washed with hot water, suspended in a considerable volume of hot water, and dissolved by adding ammonia. The solution was then treated with bone black, and on adding acetic acid to the yellow filtrate the arsonic acid separated on standing overnight. It forms crusts of spherical aggregates of microcrystals which become ochreous in color on exposure to the air. When air-dried it contains approximately 1.5 molecules of water of crystallization. The an-

[1] This Journal, **39**, 1459 (1917).

hydrous substance darkens at about 200° but does not melt below 285°, and is rather sparingly soluble in boiling water or 50% alcohol.

Subs., air-dry, 0.7072; loss, 0.0468 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{16}H_{19}O_5N_4As.1.5H_2O$: H_2O, 6.02. Found: 6.62.
Subs., anhydrous, 0.1522; (Kjeldahl) 13.90 cc. 0.1 *N* HCl. Subs., 0.2610; $Mg_2As_2O_7$, 0.0990.
Calc. for $C_{16}H_{19}O_5N_4As$: N, 13.27; As, 17.75. Found: N, 12.78; As, 18.31.

N-Phenylglycineanilide-4,4'-diarsonic Acid, $H_2O_3AsC_6H_4NHCH_2CONHC_6H_4AsO_3H_2$.—4.4 g. of arsanilic acid and 6 g. of chloroacetylarsanilic acid were dissolved in 40 cc. of *N* sodium hydroxide solution (2 mols.) and boiled for 30 minutes, during which the product began to separate. After cooling, the mixture was treated with an excess of hydrochloric acid to complete the separation. The substance was purified by redissolving in dilute alkali and reprecipitating with hydrochloric acid. It separated at once as sheaves of microscopic needles, which contain one-half molecule of water of crystallization. The anhydrous substance does not melt up to 280°. It is insoluble in boiling water and very sparingly soluble in hot 50% alcohol.

Subs., air-dry, 0.6798; loss, 0.0103 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{14}H_{16}O_7N_2As_2.0.5H_2O$: H_2O, 1.86. Found: 1.52.
Subs., anhydrous, 0.3313; (Kjeldahl) 13.4 cc. 0.1 *N* HCl; $Mg_2As_2O_7$, 0.2195.
Calc. for $C_{14}H_{16}O_7N_2As_2$: N, 5.91; As, 31.62; Found: N, 5.67; As, 31.98.

p-Acetophenyl-glycyl-p-arsanilic Acid, *p*-$CH_3COC_6H_4NHCH_2CONHC_6H_4AsO_3H_2(p)$.—2.8 g. of *p*-aminoacetophenone yielded the yellow arsonic acid after a few minutes and the heating was continued on the water bath for ½ hour. The collected solid was suspended in a moderate amount of hot water, treated with sodium hydroxide until faintly alkaline, filtered, and the acid reprecipitated from the hot solution with acetic acid. The purified product was again suspended in hot water, dissolved as above, and the solution treated with sodium acetate until the *sodium salt* of the arsonic acid began to separate. After cooling and washing with cold water the yield was 4.5 g. Recrystallized from water, in which it is sparingly soluble in the cold, the salt forms minute, narrow, pale yellow, glistening, platelets containing 3 molecules of water of crystallization.

Subs., air-dry, 1.0979; loss, 0.1258 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{16}H_{16}O_5N_2AsNa.3H_2O$: H_2O, 11.54. Found: 11.46.
Subs., anhydrous, 0.1620; 9.85 cc. N (24.0°, 762 mm.). Subs., 0.3201; $Mg_2As_2O_7$, 0.1187.
Calc. for $C_{16}H_{16}O_5N_2AsNa$: N, 6.76; As, 18.09. Found: N, 7.00; As, 17.88.

The *pure acid* was obtained from a hot, dilute solution of the sodium salt with acetic acid. It forms radiating masses of faintly yellow, microscopic needles which darken and decompose partially when heated, but do not melt below 290°. The acid is practically insoluble in boiling water and only very sparingly so in boiling 50% alcohol.

Subs., 0.1323; 8.25 cc. N (24.0°, 765 mm.).
Calc. for $C_{16}H_{17}O_5N_2As$: N, 7.15. Found: 7.22.

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AROMATIC ARSENIC COMPOUNDS. VI.

N-(Phenyl-4-Arsonic Acid)-α-Phenylglycine and its Amides.

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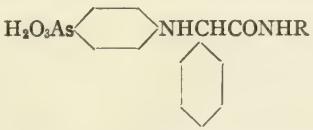
As a special extension of the general type of substances represented by the substituted amides, ureides, and anilides of the phenylglycine arsonic acids,[1] *N*-(phenyl-4-arsonic acid)-α-phenylglycine,



its amide, ureide, and a few of the substituted anilides were prepared and studied. With the exception of the glycine itself, which was obtained by hydrolysis of the amide, these substances were made from sodium arsanilate and the phenylchloroacetyl-amino compounds. It was necessary in every case to employ sodium iodide in 50% alcoholic solution in order to accelerate the condensation, since the chloro compounds alone showed little tendency to react.

The general properties of this group of substances resemble those of the simpler glycine derivatives.

EXPERIMENTAL.

N-(*Phenyl-4-arsonic Acid*)-α-*phenylglycine*, *p*-$H_2O_3AsC_6H_4NHCH(C_6H_5)CO_2H$.—Although this substance unquestionably could be prepared from arsanilic acid and α-phenylchloroacetic acid just as phenylglycine-*p*-arsonic acid has been prepared, we have obtained it by hydrolysis of the corresponding amide described below, and which we had previously prepared.

[1] This Journal, **41**, 1585 (1919).

30 g. of the sodium salt of *N*-(phenyl-4-arsonic acid)-α-phenylglycineamide were dissolved in about 5 parts of 10% sodium hydroxide solution and boiled for about 15 minutes, after which the evolution of ammonia ceased. On adding hydrochloric acid to the chilled solution until acid to congo red the free arsonic acid separated as a colorless oil which rapidly crystallized. The compound was recrystallized from a large volume of boiling water, forming lustrous, rhombic plates. The yield was 20 g. When rapidly heated to 195°, then slowly, the acid darkens and sinters, and finally effervesces at 202–3°. It is sparingly soluble in the cold in water, alcohol, or acetic acid, but dissolves more readily on warming. It is more easily soluble in methyl alcohol.

Subs., 0.2077 (Kjeldahl); 6.1 cc. 0.1 *N* HCl. Subs., 0.2674; $Mg_2As_2O_7$, 0.1168.

Calc. for $C_{14}H_{14}O_5NAs$: N, 3.99; As, 21.36. Found: N, 4.11; As, 21.10.

N-(Phenyl-4-arsonic Acid)-α-phenylglycineamide, p-$H_2O_3AsC_6H_4NHCH(C_6H_5)CONH_2$.—87 g. of arsanilic acid dissolved in 400 cc. of *N* sodium hydroxide solution, 80 g. of sodium iodide, 68 g. (1 mol.) of phenylchloroacetamide, and 500 cc. of alcohol were boiled for 4 hours. The mixture set to a solid cake on cooling. This was disintegrated and stirred with dil. hydrochloric acid until acid to congo red. The crude arsonic acid obtained in this way was purified through the sodium salt as described below. On dissolving a portion of this in water and adding acetic acid the free arsonic acid gradually separates as microscopic needles which do not melt below 280°. The substance is sparingly soluble in boiling water or 50% alcohol and practically insoluble in the cold.

Subs., 0.3260 (Kjeldahl); 19.05 cc. 0.1 *N* HCl. Subs., 0.3055; $Mg_2As_2O_7$, 0.1337.

Calc. for $C_{14}H_{15}O_4N_2As$: N, 8.00; As, 21.39. Found: N, 8.19; As, 21.12.

Sodium Salt.—The crude, washed acid was suspended in warm water, stirred, and treated with 50% sodium hydroxide solution until clear. After exactly neutralizing to litmus with acetic acid the warm solution was filtered, stirred, and treated with powdered sodium acetate until crystallization of the arsonate began. After standing in the ice-box overnight the salt was filtered off, washed first with 20%

sodium acetate solution, then with alcohol, and finally recrystallized from alcohol, forming granular aggregates of plates. The yield of air-dried salt was 76 g., the amount of water of crystallization varying from 3.5 to 5 molecules in different preparations. The salt dissolves readily in water or boiling alcohol.

Prepn. 1: Subs., air-dry, 0.3384; loss, 0.0659 *in vacuo* at 100° over H_2SO_4.
Prepn. 2: Subs., 0.6091; loss, 0.0931.
Calc. for $C_{14}H_{14}O_4N_2AsNa.3.5H_2O$: H_2O, 14.48. For 5 H_2O: H_2O, 19.48. Found: Prepn. 2: H_2O, 15.28. Prepn. 1: H_2O, 19.47.
1—Subs., anhydrous, 0.2592 (Kjeldahl); 13.80 cc. 0.1 *N* HCl.
2—Subs., anhydrous, 0.1577; 10.6 cc. N (25.5°, 758 mm.). Subs., 0.3242; $Mg_2As_2O_7$, 0.1335.
Calc. for $C_{14}H_{14}O_4N_2AsNa$: N, 7.53; As, 20.13. Found: N, 7.46, **7.67.** As, 19.88.

N-(Phenyl-4-arsonic Acid)-α-phenylglycineureide, *p*-$H_2O_3AsC_6H_4NHCH(C_6H_5)CONHCONH_2$.—4.4 g. of arsanilic acid, 5.3 g. (1.25 mols.) of phenylchloroacetyl-urea,[1] 4 g. of sodium iodide, 21 cc. of *N* sodium hydroxide solution, and 22 cc. of alcohol were boiled on the water bath for two hours. On cooling, acidifying to congo red, and rubbing, the glycineureide crystallized. The collected substance was purified by suspending in water, adding sodium hydroxide until faintly alkaline to litmus, filtering, diluting with hot water, and acidifying with acetic acid. The arsonic acid gradually separates in radiating masses of microscopic needles which contain one molecule of water of crystallization when air-dried. When rapidly heated the anhydrous substance swells and evolves gas at 195–7°. It is soluble in boiling water or 50% alcohol, but sparingly so in the cold. The yield was 3.5 g.

Subs., air-dry, 0.4267; loss, 0.0199 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{15}H_{16}O_5N_3As.H_2O$: H_2O, 4.38. Found: 4.66.
Subs., anhydrous, 0.3048 (Kjeldahl); 23.1 cc. 0.1 *N* HCl. Subs., 0.3045; $Mg_2As_2O_7$, 0.1196.
Calc. for $C_{15}H_{16}O_5N_3As$: N, 10.69; As, 19.06. Found: N, 10.62; As, 18.95.

N-(Phenyl-4-arsonic Acid)-α-phenylglycine-3′-hydroxyanilide, *p*-$H_2O_3AsC_6H_4NHCH(C_6H_5)CONHC_6H_4OH(m\text{-})$.—10.5 g. of *m*-phenylchloroacetyl-aminophenol,[2] were employed as in the previous cases.

[1] This Journal, **39**, 2433 (1917).
[2] *Ibid.*, **39**, 1445 (1917).

The resulting purple solution was diluted with water and decanted from the gummy precipitate, which was digested on the water bath with hot water. As crystallization could not be induced the mixture was set aside for about a month, when crystals began to form. The process was accelerated by warming and stirring, and the crystals were finally filtered from the hot solution and washed with hot water. The dark purple product was recrystallized twice from 50% alcohol, separating slowly as purplish, lenticular, microscopic platelets, which contain 1.5 molecules of water of crystallization when air-dry and effervesce at 155–60°. The yield was 4.5 g. When rapidly heated to 155°, then slowly, the anhydrous arsonic acid softens at about 155–60° and melts and evolves gas at about 200–10°. It is appreciably soluble in methyl alcohol, and in dilute, as well as strong alcohol. It melts in boiling water, in which it is appreciably soluble, and separates on cooling as an emulsion. It is insoluble in hot acetone.

Subs., air-dry, 0.5600; loss, 0.0333 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{20}H_{19}O_5N_2As.1.5H_2O$: H_2O, 5.76. Found: 5.95.
Subs., anhydrous, 0.0917; 5.0 cc. N (20.8°, 756 mm.). Subs., 0.3040; $Mg_2As_2O_7$, 0.1056.
Calc. for $C_{20}H_{19}O_5N_2As$: N, 6.34; As, 16.96. Found: N, 6.31; As, 16.76.

N-(Phenyl-4-arsonic Acid)-α-phenylglycine-4′-uraminoanilide, *p*-$H_2O_3AsC_6H_4NHCH(C_6H_5)CONHC_6H_4NHCONH_2$(*p*-).—The condensation of arsanilic acid and *p*-phenylchloroacetyl-aminophenyl-urea[1] (12.2 g.) was effected exactly as in the preceding example. Crystallization of the product, which started on cooling and scratching, was complete after 24 hours. After washing with 50% alcohol the substance was dissolved in dil. sodium hydroxide, the solution neutralized to litmus with acetic acid, filtered, diluted with hot water, and acidified with acetic acid. The arsonic acid separated as a gummy mass which gradually hardened and was then filtered off and washed with water. The yield was 11 g., decomposing after drying *in vacuo* at 100° at about 255° with preliminary darkening and softening. It dissolves in boiling 50% alcohol, but is very difficultly soluble in boiling water.

Subs., 0.1414; 14.2 cc. N (23.0°, 764 mm.). Subs., 0.3575; $Mg_2As_2O_7$, 0.1134.
Calc. for $C_{21}H_{21}O_5N_4As$: N, 11.57; As, 15.49. Found: N, 11.65; As, 15.31.

[1] THIS JOURNAL, **39**, 1445 (1917).

N-(Phenyl-4-arsonic Acid)-α-phenylglycine-3'-carbamidoanilide, *p*-$H_2O_3AsC_6H_4NHCH(C_6H_5)CONHC_6H_4CONH_2$(*m*-).—5.9 g. of *m*-phenylchloroacetyl-aminobenzamide,[1] gradually yielded a clear solution. After 4 hours the arsonic acid separated on rubbing, and was washed with 50% alcohol. It was then suspended in water and ammonia added in excess, and the solution treated with bone black to remove the turbidity. The filtrate was diluted to large volume with hot water and acidified with acetic acid, whereupon the acid gradually separated on srcatching in microcrystalline form and in a yield of 7 g. It darkens and softens slightly above 250°, melts with effervescence at 261–2°, and is practically insoluble in boiling water or 50% alcohol,

Subs., 0.1431; 11.3 cc. N (23.5°, 760 mm.). Subs., 0.3522; $Mg_2As_2O_7$, 0.1183.
Calc. for $C_{21}H_{20}O_5N_3As$: N, 8.96; As, 15.97. Found: N, 9.09; As, 16.20.

N-(Phenyl-4-arsonic Acid)-α-phenylglycyl-4-aminophenylacetamide, *p*-$H_2O_3AsC_6H_4NHCH(C_6H_5)CONHC_6H_4CH_2CONH_2$(*p*-).—The reaction mixture, containing 6.1 g. of α-phenylchloroacetyl-*p*-aminophenylacetamide,[1] was boiled for 3 hours, quickly becoming clear. Dilution with several volumes of water caused the deposition of a thick oil which began to crystallize after a few days. The process was hastened by warming the mixture on the water bath and rubbing, after which the substance was filtered off hot and washed with hot water. The crude product was dissolved in very dilute ammonia, treated with bone black, and the filtrate warmed on the water bath and gradually treated with an excess of acetic acid, seeding at the same time with a crystal of the crude substance and rubbing with a rod. In this way partial separation as an oil was avoided. The yield was 6 g. The arsonic acid was recrystallized from 50% alcohol, forming minute plates and flat needles which contain $\frac{1}{2}$ molecule of water of crystallization. When anhydrous it turns yellow and softens on heating, melting with decomposition at 222–3°. It is sparingly soluble in boiling water and somewhat more easily in boiling 50% alcohol.

Subs., air-dry, 0.5309; loss, 0.0086 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{22}H_{22}O_5N_3As.0.5H_2O$: H_2O, 1.62. Found: 1.82.
Subs., anhydrous, 0.1366; 10.3 cc. N (21.0°, 769 mm.). Subs., 0.3015; $Mg_2As_2O_7$, 0.0956.
Calc. for $C_{22}H_{22}O_5N_3As$: N, 8.70; As, 15.52. Found: N, 8.87; As, 15.30.

[1] This Journal, **39**, 1445 (1917).

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AROMATIC ARSENIC COMPOUNDS. VII.

Substituted Benzyl-, Phenoxyethyl-, and Phenacylarsanilic Acids.

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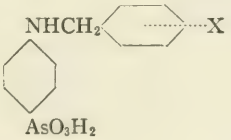
The preceding papers of this series have demonstrated that the reactivity of the amino group in arsanilic acid with chloroacetylamino compounds is such as to render possible the synthesis of a very extensive series of aromatic arsonic acids. It was thought possible that still other series, capable of similar extensive development, might be obtained by the use of other aromatic compounds containing suitable alkyl halide side chains. The present paper is a report of a few attempts in this direction which represent merely a beginning in the study of such substances, the pressure of other work having temporarily limited the development of these types of compounds.

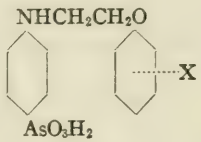
Benzyl chloride and the substituted benzyl chlorides were found to react with sodium arsanilate under suitable conditions to form benzyl and substituted benzyl-arsanilic acids,



Although long boiling of the solution in 50% alcohol was required with benzyl chloride itself in order to obtain the greatest yields, owing to the stability of the halogen in this substance, the reaction proceeded far more easily and completely in the case of the negatively substituted benzyl halides such as *p*-nitrobenzyl chloride, 3-nitro-4-hydroxybenzyl chloride, *p*-carboxybenzyl chloride, and *p*-carboxyamidobenzyl chloride, the halogen of which is very reactive. The arsonic

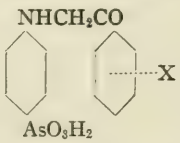
acids obtained from the above nitrobenzyl chlorides were reduced to the corresponding aminobenzyl-arsanilic acids by means of ferrous hydroxide, a method which we have found to be especially serviceable for the selective reduction of nitro-arsonic acids.[1]

Another group of substances studied was those obtained by reacting sodium arsanilate with phenoxyethyl bromide and such of its derivatives as salicylamide-bromoethyl ether and *p*-acetamino-phenoxyethyl bromide.



The reaction was found to proceed very slowly and incompletely in boiling 50% alcoholic solution even in the presence of sodium iodide which strongly accelerated the condensation when chloroacetyl compounds were in question.

Finally, the reaction between sodium arsanilate and halogen-acetyl compounds such as the phenacyl halides was studied and found to proceed in the desired sense, yielding the phenacylarsanilic acids



These compounds are yellow in color and yield yellow solutions.

All of the above groups of substances function both as acids and feeble bases, forming salts with alkalies and strong mineral acids. With the exception of the negatively substituted benzyl derivatives they are readily displaced from their alkali salts by a slight excess of acetic acid.

EXPERIMENTAL.

(*A*) *Benzyl-p-arsanilic Acids.*

Benzyl-arsanilic Acid, *p*-$C_6H_5CH_2NHC_6H_4AsO_3H_2$.—4.4 g. of arsanilic acid were dissolved in 20 cc. of *N* sodium hydroxide solution and

[1] Cf. This Journal, **40**, 1580 (1918).

boiled under a reflux condenser for 4 hours with 2.7 g. of benzyl chloride, 1.5 g. of sodium iodide, and 20 cc. of alcohol. The benzylarsanilic acid separated during the heating and was washed with 50% alcohol, and finally with ether. The yield was 4.3 g. The crude substance was dissolved in dil. sodium hydroxide, treated with bone black, and precipitated while hot with acetic acid, the arsonic acid separating quickly as flat, lustrous, microscopic needles. For analysis it was best recrystallized from hot 50% alcohol, in which it is fairly readily soluble, forming arborescent masses of microcrystals and larger prisms. The acid decomposes at about 255° with preliminary sintering, and is very sparingly soluble in hot water. Although difficultly soluble in the cold, boiling alcohol and acetic acid readily dissolve the substance. It is also soluble in methyl alcohol at room temperature and is insoluble in 10% hydrochloric acid, but stronger acid dissolves it. On adding sodium nitrite to a solution of the compound in hot acetic acid, cooling and rubbing, yellow spherules of microscopic crystals, presumably the nitroso compound, separate. On concentrating a neutral solution of the arsonic acid in aqueous sodium hydroxide to small volume and cooling, the *sodium salt* separates as glistening platelets which dissolve readily on dilution.

Subs., 0.2067; (Kjeldahl) 6.7 cc. 0.1 *N* HCl. Subs., 0.2747; $Mg_2As_2O_7$, 0.1378.

Calc. for $C_{13}H_{14}O_3NAs$: N, 4.56; As, 24.41. Found: N, 4.56; As, 24.21.

p-Nitrobenzyl-arsanilic Acid, *p*-$O_2NC_6H_4CH_2NHC_6H_4AsO_3H_2$.—8.8 g. of arsanilic acid in 40 cc. of *N* sodium hydroxide solution, 7 g. of *p*-nitrobenzyl chloride, and 40 cc. of alcohol were boiled for 3 hours, during which the molten chloride gradually disappeared. The yellow crystals which deposited on cooling were washed with 50% alcohol and then with acetone. The yield was 10 g. For purification a hot, dilute alkaline solution of the substance was precipitated with acetic acid. Recrystallized for analysis from hot 85% alcohol, in which it is sparingly soluble, it forms sheaves of flat, yellow, microscopic needles which do not melt when heated up to 280°. The acid is almost insoluble in boiling water and only sparingly so in boiling methyl alcohol or acetic acid.

Subs., 0.1598; 11.0 cc. N (22.5°, 762 mm.). Subs., 0.3023; $Mg_2As_2O_7$, 0.1320.

Calc. for $C_{13}H_{13}O_5N_2As$: N, 7.95; As, 21.28. Found: N, 7.97; As, 21.06.

p-Aminobenzyl-arsanilic Acid, p-$H_2NC_6H_4CH_2NHC_6H_4AsO_3H_2$.—40 g. of ferrous sulfate dissolved in 150 cc. of water were treated with 25% sodium hydroxide solution until the mixture remained strongly alkaline to litmus after thorough shaking.[1] Ice was added and then a solution of 7 g. of *p*-nitrobenzyl-arsanilic acid in dil. sodium hydroxide. After 5 minutes' shaking the mixture was filtered, acidification of the filtrate with acetic acid yielding the crystalline amino compound contaminated with an amorphous by-product. For purification the crude substance was dissolved in cold, dil. ammonia, leaving a small amount of amorphous material which was collected in the cold with bone black. Acidification of the clear filtrate yielded the pure amino acid at once as colorless aggregates of microscopic leaflets which decompose at about 202°. The yield was 3.5 g. The acid is practically insoluble in hot water or 50% alcohol. On boiling a solution in dil. ammonia a white, amorphous alteration product is formed which is insoluble in ammonia. This does not happen with fixed alkali. The pure acid is soluble in both alkali and mineral acids, a solution in the latter being readily diazotizable, coupling with R-salt to form a deep red dye.

Subs., 0.1510; 11.9 cc. N (21.0°, 740 mm.). Subs., 0.3241; $Mg_2As_2O_7$, 0.1573.
Calc. for $C_{13}H_{15}O_3N_2As$: N, 8.70; As, 23.28. Found: N, 8.92; As, 23.41.

3-Nitro-4-hydroxybenzyl-arsanilic Acid, 3,4-$(O_2N)HOC_6H_3CH_2NH$-$C_6H_4AsO_3H_2$(p-).—To a hot solution of 11 g. of arsanilic acid in 50 cc. of *N* aqueous sodium hydroxide a solution of 9.5 g. of 3-nitro-4-hydroxybenzyl chloride in 50 cc. of hot alcohol was added. The condensation took place rapidly but the heating was continued for 30 minutes. The alcohol was then allowed to boil off, causing the mixture to set to a thick mass of yellow crystals. These were filtered off and washed first with 50% alcohol and finally with acetone, the yield being 15 g. The crude acid was purified over the sodium salt by suspending it in a small volume of hot water and carefully treating with sodium hydroxide until solution was complete. On adding an equal volume of saturated sodium acetate solution to the reddish orange solution the *sodium salt* separated as a thick mass of long, thin, yellow, micro-

[1] *Loc. cit.*

scopic needles, which were filtered off and washed with 20% sodium acetate solution. On cautiously adding an excess of acetic acid to a hot, dilute solution of the salt the pure arsonic acid separated as aggregates of microscopic needles containing no water of crystallization. However, when recrystallized from 85% alcohol it crystallizes more slowly, forming crusts of minute, yellow crystals which contain one molecule of water of crystallization. When rapidly heated the anhydrous substance darkens and sinters above 210° and decomposes at about 245–50°. It is practically insoluble in boiling water, sparingly in the cold in alcohol, methyl alcohol, or acetic acid, and readily on boiling.

Subs., air-dry, 0.9344; loss, 0.0471 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{13}H_{13}O_6N_2As.H_2O$: H_2O, 4.66. Found: 5.04.
Subs., anhydrous, 0.2655; (Kjeldahl) 14.5 cc. 0.1 *N* HCl.
Calc. for $C_{13}H_{13}O_6N_2As$: N, 7.61. Found: 7.65.

3-Amino-4-hydroxybenzyl-arsanilic Acid.—9 g. of 3-nitro-4-hydroxybenzyl-arsanilic acid were dissolved in dil. sodium hydroxide solution and added at once to a ferrous hydroxide suspension prepared by precipitating a solution of 50 g. of ferrous sulfate with sodium hydroxide until it remained strongly alkaline on shaking. After 5 minutes' shaking the mixture was filtered on a large Büchner funnel and the dark brown filtrate acidified with acetic acid, yielding a dark brown, crystalline precipitate of the amino acid on rubbing. For purification this was dissolved in dil. hydrochloric acid and a solution of sodium acetate added as long as the precipitate which first formed redissolved on stirring. A few dark-colored flocks were collected by the addition of bone black and the faintly colored filtrate diluted and treated further with sodium acetate solution until no longer acid to congo red, the arsonic acid separating as almost colorless, microscopic platelets which contained $\frac{1}{2}$ molecule of water of crystallization. The yield was 3 g. When heated to 285° the anhydrous substance darkens slightly, but does not melt. It is sparingly soluble in boiling water or 50% alcohol. An alkaline solution darkens rapidly on standing, while an aqueous suspension gives a deep brown color with ferric chloride solution.

Subs., air-dry, 0.6106; loss, 0.0155 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{13}H_{15}O_4N_2As.0.5H_2O$: H_2O, 2.59. Found: 2.54.

Subs., anhydrous, 0.2298; (Kjeldahl) 13.45 cc. 0.1 *N* HCl. Subs., 0.2565; $Mg_2As_2O_7$, 0.1170.
Calc. for $C_{13}H_{15}O_4N_2As$: N, 8.28; As, 22.18. Found: N, 8.20; As, 22.02.

p-Carboxybenzyl-arsanilic Acid, *p*-$HO_2CC_6H_4CH_2NHC_6H_4AsO_3H_2$.—3.5 g. of *p*-carboxybenzyl chloride in 20 cc. of *N* sodium hydroxide solution were added to a solution of 4.4 g. of arsanilic acid in 20 cc. of the same medium. The mixture was gently warmed and stirred until solution was complete and then allowed to stand at room temperature, a crop of crystals soon separating. After 24 hours the crystals were filtered off, dissolved by the addition of dil. sodium hydroxide, and the solution then heated. On adding hydrochloric acid to the hot solution a gum separated which rapidly crystallized. The collected and washed solid was boiled out with alcohol to remove impurities and converted into the *sodium salt* for final purification. After dissolving in dil. sodium hydroxide solution, neutralizing with acetic acid, and concentrating to small bulk, a large volume of alcohol was added to the hot solution, causing the separation of the salt as a colorless, microcrystalline powder. Recrystallized from a concentrated aqueous solution by adding alcohol the salt formed aggregates of flat needles containing approximately $\frac{1}{2}$ molecule of water of crystallization. The yield was 2.5 g.

Subs., air-dry, 0.2886; loss, 0.0082 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{14}H_{12}O_5NAsNa_2.0.5H_2O$: H_2O, 2.23. Found: 2.84.
Subs., anhydrous, 0.3230; (Kjeldahl) 8.3 cc. 0.1 *N* HCl; $Mg_2As_2O_7$, 0.1260.
Calc. for $C_{14}H_{12}O_5NAsNa_2$: N, 3.55; As, 18.86. Found: N, 3.60; As, 18.83.

On adding acetic acid to a hot solution of the sodium salt the *free acid* separated as delicate, microscopic needles which did not melt below 280°. It is sparingly soluble in hot 50% alcohol or acetic acid and insoluble in other neutral solvents.

Subs., 0.2397; 8.4 cc. N (22.0°, 762 mm.).
Calc. for $C_{14}H_{14}O_5NAs$: N, 3.99. Found: 4.07.

p-Carboxamidobenzyl-arsanilic Acid, *p*-$H_2NCOC_6H_4CH_2NHC_6H_4AsO_3H_2$.—33 g. of arsanilic acid in 150 cc. of *N* aqueous sodium hydroxide, 25.5 g. of *p*-(ω-chloromethyl)benzamide, and 150 cc. of alcohol were boiled for two hours, a thick mass of crystals separating on cooling. After filtering and washing first with water and then with alco-

hol, the arsonic acid was converted into the *sodium salt*. The solution of the acid in a small volume of warm, dil. sodium hydroxide was carefully neutralized with acetic acid. Separation of the salt, which commenced on cooling, was completed by adding saturated sodium acetate solution. Recrystallized from 50% alcohol the salt forms aggregates of thin plates containing 2.5 molecules of water of crystallization and is easily soluble in water. The yield was 30 g.

Subs., air-dry, 0.6617; loss, 0.0732 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{14}H_{14}O_4N_2AsNa.2.5H_2O$: H_2O, 10.79. Found: 11.08.
Subs., anhydrous, 0.3216; (Kjeldahl) 17.0 cc. 0.1 *N* HCl; $Mg_2As_2O_7$, 0.1329.
Calc. for $C_{14}H_{14}O_4N_2AsNa$: N, 7.53; As, 20.13. Found: N, 7.41; As, 19.95.

Acetic acid added to a hot solution of the salt precipitates the *arsonic acid* as sheaves and plumes of microscopic needles. It does not melt below 280° and is almost insoluble in boiling water, 50% alcohol, or methyl alcohol.

Subs., 0.2308: (Kjeldahl) 13.1 cc. 0.1 *N* HCl.
Calc. for $C_{14}H_{15}O_4N_2As$: N, 8.00. Found: 7.95.

(*B*) *Phenoxyethyl- and Phenacyl-p-arsanilic Acids.*

Phenoxyethyl-arsanilic Acid, *p*-$C_6H_5OCH_2CH_2NHC_6H_4AsO_3H_2$.— Phenoxyethyl bromide was found to react very slowly with arsanilic acid, and although condensation was somewhat accelerated by the addition of sodium iodide, long-continued boiling of the reaction mixture was found necessary. A solution of 4.4 g. of arsanilic acid in 20 cc. of *N* aqueous sodium hydroxide, 4 g. of sodium iodide, 4 g. of phenoxyethyl bromide, and 20 cc. of alcohol were boiled for 24 hours. On cooling and diluting with water the reaction product crystallized as glistening leaflets which were filtered off and washed first with water and then with ether to remove unchanged bromide. The yield was 3 g. The acid was converted into the *sodium salt* by dissolving in a small amount of dil. sodium hydroxide solution, exactly neutralizing to litmus with acetic acid, and then treating with dry acetone until a turbidity was produced. On standing in the refrigerator the salt slowly separated as delicate needles. Recrystallized from a small volume of 85% alcohol it forms flat, microscopic needles containing 3.5 molecules of water of crystallization.

Subs., air-dry, 0.7668; loss, 0.1119 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{14}H_{15}O_4NAsNa.3.5H_2O$: H_2O, 14.89. Found: 14.59.

On treating the hot solution of the salt with acetic acid the free *arsonic acid* separated as glistening scales containing one molecule of water of crystallization. It is very sparingly soluble in boiling water or acetic acid, but is appreciably soluble in hot methyl alcohol, alcohol, or 50% alcohol. The anhydrous substance does not decompose below 280°.

Subs., air-dry, 0.4534; loss, 0.0242 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{14}H_{16}O_4NAs.H_2O$: H_2O, 5.07. Found: 5.34.
Subs., anhydrous, 0.2263; (Kjeldahl) 7.0 cc. 0.1 *N* HCl. Subs., 0.2569; $Mg_2As_2O_7$, 0.1190.
Calc. for $C_{14}H_{16}O_4NAs$: N, 4.16; As, 22.22. Found: N, 4.33; As, 22.37.

p-Acetamino-phenoxyethyl-arsanilic Acid, *p*-$CH_3CONHC_6H_4OCH_2$-$CH_2NHC_6H_4AsO_3H_2$.—Arsanilic acid was condensed as in the previous example with *p*-acetamino-phenoxyethyl bromide. The reaction mixture was diluted with water, made alkaline with sodium hydroxide, filtered from unchanged halide, and acidified with acetic acid, yielding the crystalline arsonic acid. This was converted into the *sodium salt* by solution in a small volume of dil. sodium hydroxide, careful neutralization with acetic acid, and addition of saturated sodium acetate solution. After standing in the refrigerator the sodium salt was filtered off and washed with 20% sodium acetate solution and finally with 85% alcohol. Purified by dissolving in a small volume of water and precipitating with several volumes of alcohol, the salt forms colorless, microscopic platelets which contain about 3 molecules of water of crystallization.

Subs., air-dry, 0.5364; loss, 0.0580 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{16}H_{18}O_5N_2AsNa.3H_2O$: H_2O, 11.49. Found: 10.81.
Subs., anhydrous, 0.3250; (Kjeldahl) 15.6 cc. 0.1 *N* HCl; $Mg_2As_2O_7$, 0.1225.
Calc. for $C_{16}H_{18}O_5N_2AsNa$: N, 6.73; As, 18.01. Found: N, 6.72; As, 18.20.

On treating a hot solution of the salt with acetic acid the *arsonic acid* separated as delicate, glistening needles and platelets. Recrystallized from 50% alcohol it forms long, thin, narrow plates which do not melt below 275°. It is practically insoluble in boiling water and very difficultly so in hot alcohol or methyl alcohol, but dissolves in hot 50% alcohol or glacial acetic acid.

Subs., 0.2075; (Kjeldahl) 14.95 cc. 0.0714 *N* HCl.
Calc. for $C_{16}H_{19}O_5N_2As$: N, 7.11. Found: 7.21.

o-Carboxamido-phenoxyethyl-arsanilic Acid, *o*-$H_2NCOC_6H_4OCH_2CH_2$-$NHC_6H_4AsO_3H_2$.—The reaction mixture, similarly obtained from 10 g. of salicylamide-bromoethyl ether (2-bromoethoxy-benzamide)[1] was diluted with water, made alkaline with sodium hydroxide, filtered, and the filtrate acidified with acetic acid, depositing an amorphous, gelatinous precipitate followed by crystals on long standing in the refrigerator. The product was recrystallized from a small volume of hot 50% alcohol, 4 g. of the arsonic acid separating in wedge-shaped, microscopic prisms. For analysis it was again recrystallized from 50% alcohol. The substance is very difficultly soluble in boiling water, and when pure is only sparingly soluble in most hot solvents except hot acetic acid and hot 50% alcohol. When precipitated from its solution in alkali by means of acetic acid it separates slowly as rosets of broad, microscopic needles. When rapidly heated it does not melt below 280°, but if the temperature is held at this point the acid slowly melts and decomposes.

Subs., 0.2930; (Kjeldahl) 15.30 cc. 0.1 *N* HCl; $Mg_2As_2O_7$, 0.1185.
Calc. for $C_{15}H_{17}O_5N_2As$: N, 7.37; As, 19.62. Found: N, 7.32; As, 19.52.

Phenacylarsanilic Acid, $C_6H_5COCH_2NHC_6H_4AsO_3H_2$.—4.4 g. of arsanilic acid in 20 cc. of *N* aqueous sodium hydroxide and a solution of 4 g. of μ-bromoacetophenone in 20 cc. of alcohol were boiled for one hour, the arsonic acid crystallizing during the reaction. The yield was 4 g. It was recrystallized by dissolving in dil. sodium hydroxide solution, heating and acidifying, the phenacyl compound separating as faintly yellow, arborescent aggregates of delicate, microscopic needles. It decomposes at 185–7° with preliminary softening and is sparingly soluble in the usual solvents. An alkaline solution is yellow, the color deepening on standing. Conc. sulfuric acid dissolves the substance with a bright yellow color.

Subs., 0.1711; 6.4 cc. N (21.0°, 766 mm.). Subs., 0.3519; $Mg_2As_2O_7$, 0.1625.
Calc. for $C_{14}H_{14}O_4NAs$: N, 4.18; As, 22.36. Found: N, 4.38; As, 22.29.

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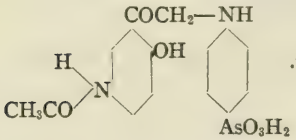
[1] *J. Biol. Chem.*, **21**, 449 (1915).

2-Hydroxy-5-acetamino-phenacylarsanilic Acid,



.—A warm solution of 4.4 g. of arsanilic acid in 10 cc. of 2 *N* sodium hydroxide and 10 cc. of alcohol was treated with 5.5 g. of 3-acetamino-6-hydroxyphenacyl bromide.[1] The bromide dissolved rapidly and the mixture was heated on the water bath for an hour. On dilution with water an amorphous precipitate was formed, followed on standing in the refrigerator by a crystalline substance. The precipitate, washed with water and acetone, could not be recrystallized directly from neutral solvents, but purification was finally effected by taking advantage of the weakly basic properties of the arsonic acid. A suspension in 1:1 hydrochloric acid was treated with conc. hydrochloric acid until most of the substance had dissolved, then rapidly filtered, and the dark yellow filtrate treated with several volumes of hot water. Owing to the hydrolysis of its hydrochloride the phenacyl compound separated at once as a dark yellow powder consisting of aggregates of microscopic platelets which redden and decompose at 228°. The yield was only 1.2 g. The acid is practically insoluble in hot water or 50% alcohol and dissolves in conc. sulfuric acid with a reddish brown color.

Subs., 0.2946; (Kjeldahl) 14.5 cc. 0.1 *N* HCl; $Mg_2As_2O_7$, 0.1124.
Calc. for $C_{10}H_{17}O_6N_2As$: N, 6.86; As, 18.36. Found: N, 6.89; As, 18.43.

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[1] *3-Acetamino-6-hydroxyphenacyl Bromide.*—From acetophenetidine, bromoacetyl chloride, and aluminum chloride with exclusion of moisture, similarly to the corresponding chloro compound [Kunckell, *Ber.*, **34**, 128 (1901)]. The crude product (95% of the theory) was used for the preparation of the arsonic acid. A portion of the crude bromide was boiled with chloroform and the filtrate treated with ligroin, the bromide separating as a drab-colored precipitate melting at 133–5° with preliminary softening and slight gas evolution to a turbid, red-brown liquid which clears at 136°. The bromide dissolves readily in acetone or alcohol, less easily in boiling chloroform. The yellowish solution in alcohol changes to a deep olive green on addition of a drop of ferric chloride solution, or to orange-red on addition of aqueous sodium hydroxide.

Subs., 0.1785; 7.85 cc. N (21.0°, 758 mm.). Subs., 0.1673; (Carius) AgBr, 0.1179.
Calc. for $C_{10}H_{10}O_3NBr$: N, 5.15; Br, 29.37. Found: N, 5.09; Br, 29.99.

[Reprinted from The Journal of the American Chemical Society, November, 1919, Vol. xli, No. 11, pp. 1834–1840.]

AROMATIC ARSENIC COMPOUNDS. VIII.

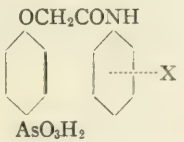
The Amides of (4-Arsonic Acid)-Phenoxyacetic Acid and the Isomeric Phenoxyacetyl-Arsanilic Acids.

By WALTER A. JACOBS and MICHAEL HEIDELBERGER.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Received July 2, 1919.

The preparation of *o*-phenylglycollic acid *p*-arsonic acid ([4-arsonic acid]-phenoxyacetic acid) by the action of sodium chloroacetate on sodium *p*-hydroxyphenyl-arsonate in boiling aqueous solution has been described in German patent 216,270. We have found that chloroacetylamino compounds also react with sodium *p*-hydroxyphenyl-arsonate to give the substituted amides of phenylglycollic acid *p*-arsonic acid with the following general formula:



In this reaction a condition for the success of the condensation was the addition of an extra molecule of sodium hydroxide in order to form the sodium phenolate, as in the preparation of phenyl ethers with alkyl halides in general. When using chloroacetyl compounds which were sufficiently stable in alkaline solution the yields of the desired products were good, but in those cases in which the halide was readily decomposed the yields suffered accordingly.

In general, the arsonic acids of this type crystallize readily when pure, possess high decomposition points, and are sparingly soluble in the usual solvents. They are stronger acids than the amides of phenylglycinearsonic acid, since only mineral acids or a large excess of acetic acid displace them completely from their salts.

In a preceding paper it was shown that the sodium salt of chloroacetyl-arsanilic acid reacted smoothly with amines with the formation

of substituted glycyl-arsanilic acids.[1] On replacing the amine by a phenolic compound the substituted phenoxyacetyl or phenyl ether glycollyl-arsanilic acids were obtained with the general formula:

These substances are of course isomeric with the amides of (4-arsonic acid)-phenoxyacetic acid. As in the preparation of the latter substances an extra molecule of alkali was required for the conversion of the phenolic compound into the phenolate. Since the reaction of the mixture was, therefore, strongly alkaline, the sensitive chloroacetyl-arsanilic acid suffered partial decomposition, so that the yields of the desired phenoxyacetyl compounds were poorer than in the synthesis of the glycyl-arsanilic acids.

As a rule the phenoxyacetyl-arsanilic acids crystallize readily and are sparingly soluble in the usual solvents, but yield readily soluble sodium salts. They are stronger acids than the glycyl-arsanilic acids and are completely displaced from their salts only by mineral acid or a large excess of acetic acid.

On reduction both of these groups of substances yield arsinoxides and arseno compounds, substances which will be described when our studies with the group are completed.

EXPERIMENTAL.

(*A*) *Derivatives of (4-Arsonic Acid)-phenoxyacetic Acid.*

(4-Arsonic Acid)-phenoxyacetic Methyl Ester, *p*-$H_2O_3AsC_6H_4OCH_2CO_2CH_3$.—10 g. of (4-arsonic acid)-phenoxyacetic acid[2] were refluxed for two hours with a mixture of 30 g. of dry methyl alcohol and 3 g. of conc. sulfuric acid. On dilution with water the ester separated in a yield of 9.5 g. Recrystallized from hot water it forms lustrous plates which, when rapidly heated, partially melt with gas evolution at about 192–5° and then gradually decompose at a higher temperature without melting completely. The ester is readily soluble in methyl alcohol, hot ethyl alcohol, or water, but sparingly in cold alcohol or water.

[1] This JOURNAL, 41, 1809 (1919).
[2] Ger. pat. 216,270.

Subs., 0.2967; $Mg_2As_2O_7$, 0.1575.
Calc. for $C_9H_{11}O_6As$: As, 25.85. Found: 25.62.

(4-Arsonic Acid)-phenoxyacetamide, p-$H_2O_3AsC_6H_4OCH_2CONH_2$.—60 g. of the methyl ester were added slowly, with chilling and shaking, to 360 cc. of conc. ammonia. During the addition the mixture set to a thick mass of what appeared to be the ammonium salt of the unchanged ester and this had to be broken up occasionally during the further addition of the ester. When all had been added the mixture was vigorously shaken to disintegrate the mass as thoroughly as possible and then allowed to stand at room temperature for 24 hours. The ammonium salt changed definitely in character, presumably due to the formation of the ammonium salt of the amide. The mixture was finally diluted with water until solution was complete and the excess of ammonia removed *in vacuo*, after which the solution was made faintly acid to congo red with hydrochloric acid, causing the immediate separation of the arsonic acid. On recrystallization from hot water the amide separated as rhombic, microscopic prisms. The yield was 37.5 g. The compound does not melt below 280° and is sparingly soluble in cold water or hot alcohol, but easily soluble in boiling water.

Subs., 0.1312; 6.1 cc. N (20.5°, 750 mm.)
Calc. for $C_8H_{10}O_5NAs$: N, 5.09. Found: 5.34.

Sodium Salt.—A solution of the acid in dil. aqueous sodium hydroxide was carefully neutralized with acetic acid and concentrated to small volume *in vacuo*. On adding alcohol the salt separated slowly as glistening platelets which contained no water of crystallization and were easily soluble in water.

Subs., 0.1371; 5.7 cc. N (22.5°, 765 mm.). Subs., 0.3120; $Mg_2As_2O_7$, 0.1617.
Calc. for $C_8H_9O_5NAsNa$: N, 4.71; As, 25.23. Found: N, 4.84; As, 25.00.

(4-Arsonic Acid)-phenoxyacetanilide, p-$H_2O_3AsC_6H_4OCH_2CONHC_6H_5$.—5 g. of sodium p-hydroxyphenyl-arsonate,[1] 3.4 g. of chloroacetanilide,[2] and 3 g. of sodium iodide were boiled in a mixture of 20 cc. of N aqueous sodium hydroxide and 20 cc. of alcohol for 3 hours. The alcohol was then allowed to boil off and the residue acidified

[1] This Journal, **41**, 1447 (1919).
[2] *Ibid.*, **39**, 1441 (1917).

with hydrochloric acid, resulting in a gummy precipitate which rapidly recrystallized. For purification the collected material was dissolved in dil. sodium hydroxide and the filtrate treated, preferably while hot, with an excess of hydrochloric acid. The arsonic acid separated at once as glistening platelets. When rapidly heated the anilide darkens slightly above 250° but does not melt below 280°. From hot 50% alcohol, in which it is fairly readily soluble, it separates as minute prisms and masses of thin, microscopic plates. Although but sparingly soluble in the cold it is appreciably soluble in hot water, alcohol, and acetic acid. Cold methyl alcohol also dissolves the substance.

Subs., 0.3024; (Kjeldahl) 8.6 cc. 0.1 *N* HCl. Subs., 0.3455; $Mg_2As_2O_7$, 0.1530. Calc. for $C_{14}H_{14}O_5NAs$: N, 3.99; As, 21.34. Found: N, 3.98; As, 21.37.

(4-Arsonic Acid)-phenoxyacetyl-3-aminophenol, *p*-$H_2O_3AsC_6H_4OCH_2CONHC_6H_4OH$(*m*-).—43.6 g. of *p*-hydroxyphenyl-arsonic acid[1] dissolved in 400 cc. of *N* sodium hydroxide solution, and 38 g. of *m*-chloroacetyl-aminophenol[2] were boiled for 15 minutes. During the heating an amorphous substance separated and more of this material was precipitated on dilution with water. The mixture was made definitely acid with acetic acid and cleared by treating with bone black in the cold. On the addition of hydrochloric acid to the filtrate until acid to congo red a pinkish paste separated, but on standing and scratching this was followed by crystals. After 24 hours crystallization was complete. The nitrogen figures obtained with this substance were nearly 1% high and the arsenic figures too low, indicating the presence of a contaminating arsonic acid containing a higher percentage of nitrogen. It is possible that the hydroxyl group in the *m*-aminophenol may have reacted with a second molecule of *m*-chloroacetyl-aminophenol giving a more complex arsonic acid. Since direct recrystallization of the crude product from a suitable solvent such as 50% alcohol or 50% acetic acid did not remove this contaminating material it was found necessary to pass through the *sodium salt*, which was fortunately found to have suitable properties.

[1] THIS JOURNAL, **41**, 1446 (1919).

[2] *Ibid.*, **39**, 1442 (1917). An improved method will be published shortly.

The crude acid was suspended in a small volume of water and dissolved by the addition of sodium hydroxide solution. After neutralization with acetic acid an equal volume of saturated salt solution was added. On scratching, the pink solution gradually deposited the sodium salt, which carried the color with it. After standing in the refrigerator it was filtered off and washed with 15% salt solution. The crude salt was recrystallized from a small volume of hot water, separating as a mass of pink, lustrous, microscopic needles and long, thin platelets. On dissolving in hot water and adding hydrochloric acid a milky emulsion was formed which almost immediately crystallized. The free acid settled quickly as a heavy, sandy powder consisting of aggregates of irregular, microscopic leaflets to which the pink color still adhered. When rapidly heated it melts and decomposes at 238–40°. It is very sparingly soluble in boiling water or boiling acetic acid but dissolves more readily in hot 50% alcohol.

Subs., 0.2067; (Kjeldahl) 5.85 cc. 0.1 *N* HCl. Subs., 0.3209; $Mg_2As_2O_7$, 0.1375. Calc. for $C_{14}H_{14}O_6NAs$: N, 3.83; As, 20.42. Found: N, 3.97; As, 20.68.

(*4-Arsonic Acid*)-*phenoxyacetyl-4-aminophenol.*—22 g. of *p*-hydroxyphenyl-arsonic acid dissolved in 100 cc. of 2 *N* sodium hydroxide solution (2 mols.), and 19 g. of *p*-chloroacetyl-aminophenol[1] were boiled under a reflux condenser. A precipitate separated from the clear solution after a few seconds and was probably an alteration product of the chloroacetyl compound. After 30 minutes the mixture was considerably diluted, acidified with acetic acid, and treated with bone black in the cold. The clear filtrate yielded a crystalline arsonic acid on acidification to congo red with hydrochloric acid and scratching. After 24 hours the collected substance was repurified by reprecipitation of its solution in dil. ammonia with hydrochloric acid. As the analysis showed the product to be high in nitrogen it was finally purified over the *sodium salt.*

The acid was suspended in a small volume of hot water and carefully treated with sodium hydroxide solution until dissolved. On cooling, the colorless sodium salt crystallized and was filtered off, washed, redissolved in hot water, and acidified with hydrochloric acid. The arsonic acid separated at once as curved, colorless, micro-

[1] This Journal, **39**, 1442 (1917).

scopic crystals. When rapidly heated it gradually darkens and decomposes at 238–40°. It is very difficultly soluble in boiling water, alcohol, or methyl alcohol, but dissolves more easily in hot 50% alcohol. On adding a few drops of sodium nitrite solution to a suspension of the acid in hot acetic acid a clear, orange solution is obtained which deposits spherules of yellow crystals, probably a nitroso compound, in cooling.

Subs., 0.2113; (Kjeldahl) 6.15 cc. 0.1 *N* HCl. Subs., 0.3269; $Mg_2As_2O_7$, 0.1354. Calc. for $C_{14}H_{14}O_6NAS$: N, 3.83; As, 20.42. Found: N, 4.07; As, 20.00.

(4-Arsonic Acid)-phenoxyacetyl-4-aminophenylurea, *p*-$H_2O_3AsC_6H_4O$-$CH_2CONHC_6H_4NHCONH_2$(*p*-).—8.8 g. of *p*-hydroxyphenyl-arsonic acid in 40 cc. of 2 *N* sodium hydroxide solution, 8 g. of sodium iodide, 9.2 g. of *p*-chloroacetyl-aminophenylurea,[1] and 40 cc. of alcohol were refluxed for two hours. After dilution with water an excess of acetic acid was added. On scratching a white powder separated and this was filtered off, dissolved in dil. ammonia, and treated while hot with an excess of acetic acid. On cooling the arsonic acid separated as aggregates of microscopic needles in a yield of 7 g. When rapidly heated the substance darkens and softens at about 230–40° but does not melt entirely up to 265°. It is practically insoluble in boiling water or 50% alcohol.

Subs., 0.1322; 12.0 cc. N (26.0°, 757 mm.). Subs., 0.3605; $Mg_2As_2O_7$, 0.1368. Calc. for $C_{15}H_{16}O_6N_3As$: N, 10.26; As, 18.32. Found: N, 10.33; As, 18.32.

(*B*) *Derivatives of Phenoxyacetyl-p-Arsanilic acid.*

Phenoxyacetyl-arsanilic Acid, $C_6H_5OCH_2CONHC_6H_4AsO_3H_2$(*p*-).—4.4 g. of arsanilic acid were dissolved in 50 cc. of 20% sodium acetate solution and treated, while chilling and shaking, with 4 g. of phenoxyacetyl chloride. The reaction product separated at once. After making acid to congo red with hydrochloric acid the substance was filtered off, washed with water, and finally with ether. The yield was 3.5 g. Recrystallized from hot 50% alcohol, in which it is fairly readily soluble, the pure arsonic acid separates slowly as colorless, microscopic crystals which are soluble in boiling methyl alcohol or

[1] THIS JOURNAL, **39, 1456 (1917).**

acetic acid, and very sparingly so in boiling water. It darkens slightly above 250° but does not decompose below 280°.

Subs., 0.3120; (Kjeldahl) 8.95 cc. 0.1 *N* HCl; $Mg_2As_2O_7$, 0.1370.
Calc. for $C_{14}H_{14}O_5NAs$: N, 3.99; As, 21.34. Found: N, 4.02; As, 21.19.

The acid was also prepared, although in poor yield, by boiling a solution of 3 g. of chloroacetyl-arsanilic acid[1] in 20 cc. of *N* aqueous sodium hydroxide with 1 g. of phenol under an air condenser for ½ hour. The substance was rapidly precipitated on cooling and adding acetic acid and was recrystallized from 50% alcohol. It separated slowly as wedge-shaped plates and prisms which showed the same properties as when made by the first method.

Subs., 0.2347; $Mg_2As_2O_7$, 0.1052.
Calc. for $C_{14}H_{14}O_5NAs$: As, 21.34. Found: 21.63.

p-Oxamino-phenoxyacetyl-arsanilic Acid, *p*-$HO_2CCONHC_6H_4OCH_2$-$CONHC_6H_4AsO_3H_2$(*p*-).—In an attempt to prepare the amide of this acid by starting with *p*-hydroxy-oxanilamide, the conditions of the experiment caused the cleavage of the amide group with the formation of the carboxylic acid.

Equivalent amounts of chloroacetyl-arsanilic acid and *p*-hydroxy-oxanilamide were dissolved separately in theoretical amounts of *N* sodium hydroxide solution and mixed, an immediate precipitation of the phenolic compound occurring. The mixture was boiled for 20 minutes, after which it reacted feebly acid, and the unchanged amide was filtered off and the filtrate acidified with hydrochloric acid. The resulting precipitate was dissolved in very dilute ammonia and the hot filtrate acidified with acetic acid. The cream-colored, microscopic crystals which separated proved to be the free carbonic acid formed by saponification of the amide in the boiling alkaline solution.

The substance darkens but does not melt below 280°. It is appreciably soluble in boiling water or 50% alcohol and separates from water with one molecule of water of crystallization.

Subs., air-dry, 0.4425; loss, 0.0196 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{16}H_{15}O_8N_2As.H_2O$: H_2O, 3.95. Found: 4.43.
Subs., anhydrous, 0.1610; 9.15 cc. N (23.5°, 753 mm.). Subs., 0.3577; $Mg_2As_2O_7$, 0.1293.
Calc. for $C_{16}H_{15}O_8N_2As$: N, 6.39; As, 17.10. Found: N, 6.48; As, 17.45.

[1] This Journal, **41**, 1810 (1919).

p-Uramino-phenoxyacetyl-arsanilic Acid, *p*-$H_2NCONHC_6H_4OCH_2$-$CONHC_6H_4AsO_3H_2$(*p*-).—11.8 g. of chloroacetyl-arsanilic acid, 6.5 g. of *p*-hydroxyphenylurea, and 40 cc. of 2 *N* sodium hydroxide solution were refluxed for ½ hour. On cooling the *sodium salt* of the reaction product crystallized and the precipitation was completed by the addition of an equal volume of saturated sodium acetate solution. This salt was filtered off, washed with 20% sodium acetate solution, and finally with alcohol. For purification the crude product was dissolved in a small volume of water, treated with bone black, and then with alcohol until crystallization began. After filtration it was washed with 85% alcohol. The yield was 3.5 g., crystallizing as radiating masses of minute needles which contain approximately 3 molecules of water of crystallization.

Subs., air-dry, 0.5095; loss, 0.0531 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{15}H_{15}O_6N_3AsNa.3H_2O$: H_2O, 11.13. Found: 10.42.
Subs., anhydrous, 0.3510; (Kjeldahl) 23.5 cc. 0.1 *N* HCl; $Mg_2As_2O_7$, 0.1240.
Calc. for $C_{15}H_{15}O_6N_3AsNa$: N, 9.75; As, 17.38. Found: N, 9.39; As, 17.05.

The free *arsonic acid* separates from the hot solution of the salt on adding acetic acid as aggregates of microscopic spindles which are practically insoluble in boiling water or 50% alcohol. It decomposes at about 280–3° with preliminary darkening.

Subs., 0.1332; 11.75 cc. N (29.0°, 765 mm.).
Calc. for $C_{15}H_{16}O_6N_3As$: N, 10.27. Found: 10.04.

o-Carboxamido-phenoxyacetyl-arsanilic Acid, *o*-$H_2NCOC_6H_4OCH_2$-$CONHC_6H_4AsO_3H_2$(*p*-).—The reaction mixture from 11.2 g. of salicylamide was diluted to about 250 cc. and treated with sodium carbonate solution until just alkaline to litmus. The filtrate from the unchanged salicylamide was acidified with acetic acid, and on scratching the arsonic acid separated as fine needles. The crude acid was dissolved in a small volume of dil. sodium hydroxide and carefully neutralized with acetic acid. On adding an equal volume of saturated sodium acetate to the filtered solution the *sodium salt* quickly separated, forming a thick paste of crystals. The salt was washed with 20% sodium acetate solution and finally with alcohol and recrystallized from 85% alcohol, separating as prismatic needles containing approximately 5.5 molecules of water of crystallization.

Subs., air-dry, 0.5945; loss, 0.1177 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{15}H_{14}O_6N_2AsNa.5.5H_2O$: H_2O, 19.23. Found: 19.79.
Subs., anhydrous, 0.3026; (Kjeldahl) 14.5 cc. 0.1 *N* HCl; $Mg_2As_2O_7$, 0.1120.
Calc. for $C_{15}H_{14}O_6N_2AsNa$: N, 6.73; As, 18.02. Found: N, 6.71; As, 17.87.

On acidifying a hot solution of the sodium salt with acetic acid the free *arsonic acid* separates as delicate needles which do not decompose below 280°. It is appreciably soluble in boiling 50% alcohol or acetic acid, but does not dissolve readily in the other hot solvents.

Subs., 0.2082; (Kjeldahl) 10.4 cc. 0.1 *N* HCl.
Calc. for $C_{15}H_{15}O_6N_2As$: N, 7.11. Found: 7.00.

p-Carboxamido-phenoxyacetyl-arsanilic Acid.—2.8 g. of *p*-hydroxy-benzamide yielded, on cooling, a thick crystalline mass of the *sodium salt.* This was dissolved by dilution with warm water and the solution filtered from unchanged *p*-hydroxy-benzamide, after which the arsonic acid was precipitated as a colorless powder on acidification with acetic acid. This was reconverted into the sodium salt by dissolving in a small volume of dil. sodium hydroxide, carefully neutralizing with acetic acid, and salting out with saturated sodium acetate solution. The product so obtained was then recrystallized from a small volume of hot water, separating as rosets of long, flat, delicate needles which contained approximately 7.5 molecules of water of crystallization. The yield was 3.7 g.

Subs., air-dry, 0.4550; loss, 0.1097 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{15}H_{14}O_6N_2AsNa.7.5H_2O$: H_2O, 24.52. Found: 24.10.
Subs., anhydrous, 0.1547; 9.3 cc. N (27.0°, 758 mm.). Subs., 0.2994; $Mg_2As_2O_7$, 0.1117.
Calc. for $C_{15}H_{14}O_6N_2AsNa$: N, 6.73; As, 18.02. Found: N, 6.82; As, 18.00.

On adding acetic acid to a hot solution of the sodium salt the free *arsonic acid* separated slowly as long, glistening needles which do not melt below 280°, and are practically insoluble in the usual boiling solvents.

Subs., 0.1495; (Kjeldahl) 7.6 cc. 0.1 *N* HCl.
Calc. for $C_{15}H_{15}O_6N_2As$: N, 7.11. Found: 7.12.

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CHEMOTHERAPY OF TRYPANOSOME AND SPIROCHETE INFECTIONS.

CHEMICAL SERIES. I.

N-PHENYLGLYCINEAMIDE-*p*-ARSONIC ACID.

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(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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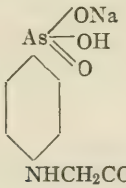
For a number of years the writers, in conjunction with Dr. Wade H. Brown and Dr. Louise Pearce, have been engaged in the synthesis of certain new types of organic arsenic compounds for the treatment of experimental trypanosome and spirochete infections. Although considerable of our effort has been given to the study of trivalent arsenicals our attention was at first confined to the development of compounds containing arsenic in the pentavalent form as presented by the arsonic acids. This group seemed best suited for the synthetic procedure which was employed and afforded the best opportunity for obtaining such information as to the relationship between chemical structure and biological action as would be useful for further work. Again it was felt that if a practicable and efficient drug could be found within this group it would go far toward eliminating certain of the well known practical disadvantages of the usual arseno compounds, for as an arsonic acid it should form soluble and stable salts and perhaps offer fewer of the preparative uncertainties than have been the experience with trivalent arsenicals.

In the course of these studies a number of substances have been prepared which have given interesting experimental results. However, those obtained with one in particular, the sodium salt of *N*-phenylglycineamide-*p*-arsonic acid



were such as to demand special attention. The simplicity of this compound, the ease of preparing it, its relatively inexpensive character, stability, and solubility, and its favorable biological behavior, warrant a publication of results. We wish therefore to present in conjunction with the following biological papers of Dr. Brown and Dr. Pearce, the facts as to the preparation and properties of the substance which will be appropriate in the present place. A somewhat fuller description of the substance and of related compounds has appeared elsewhere.[1]

Phenylglycine-*p*-arsonic acid, and its homologue, *o*-methylphenylglycine-*p*-arsonic acid, have already been described in German Patent, No. 204,664. These substances, of interest solely as the source of the arsenophenylglycines which were obtained by their reduction, were readily prepared by the interaction of the sodium salts of the amino-arylarsonic acids and sodium chloroacetate. In our investigations we have found that a reaction of this type, with α-halogenacylamino compounds, XCH_2CONHR, instead of the α-halogen acids, XCH_2COOH, is capable of practically unlimited extension and has rendered possible the preparation of a new series of aromatic arsenic compounds. In these substances the free carboxyl group of the above glycines has been changed to the amide or substituted amide group.

N-Phenylglycineamide-*p*-arsonic acid,[2] prepared by the methods to be described in the experimental part, readily yields a colorless,

[1] Jacobs, W. A., and Heidelberger, M., *J. Am. Chem. Soc.*, 1919, xli, 1587.

[2] This substance and related compounds to be described elsewhere are covered by United States Patents, Nos. 1,280,119–1,280,127. Patents have also been applied for in foreign countries.

All discoveries made at The Rockefeller Institute for Medical Research are made freely available to the public, in accordance with the philanthropic purposes of the institution. In order to insure purity of product and protection against exploitation, it has been deemed necessary in certain instances to protect the

crystalline sodium salt which is extremely easily soluble in water, forming neutral solutions, which are perfectly stable. In fact a 10 per cent solution may be boiled a reasonable length of time without appreciable cleavage of ammonia or arsenic.

The materials required and the method of preparation are such that the large scale production of the substance should offer little difficulty.

EXPERIMENTAL.

N-(Phenyl-4-Arsonic Acid)-Glycineamide (N-Phenylglycineamide-p-Arsonic Acid).—Of the two methods used for the preparation of this substance that described first is more direct.

434 gm. of arsanilic acid were dissolved in 2 liters of normal sodium hydroxide solution. After the addition of 375 gm. of chloroacetamide the mixture was boiled under a reflux condenser for 45 minutes, the clear solution setting to a solid mass of the crude product on cooling. 75 cc. of concentrated hydrochloric acid were added to the cold mixture to hold any unchanged arsanilic acid in solution and the substance was then filtered off and carefully washed with cold water. For purification it was suspended in sufficient water to form a thin paste and carefully treated, with stirring, with 25 per cent sodium hydroxide solution until the acid was completely dissolved. The filtered solution was then treated with an excess of acetic acid whereupon the substance separated as minute, lustrous plates. After filtering, washing thoroughly, and drying, the yield was 300 gm.

The acid is very sparingly soluble in cold water but dissolves readily on heating. It separates from the hot aqueous solution in aggregates of long, thin plates. It is insoluble in methyl alcohol, acetone, or chloroform and sparingly in hot methyl or ethyl alcohol, but dissolves in boiling acetic acid. It is sparingly soluble in dilute

discoveries by patents. It is the purpose of the Institute to permit any drugs which may prove of practical therapeutic value to be manufactured under license by suitable chemical firms and under conditions of production which will insure the biological qualities of the drugs and their marketing at reasonable prices. Other than through the issuance of license, The Rockefeller Institute does not participate in any way in the commercial preparation or sale of the manufactured chemicals; and it receives no royalties or other pecuniary benefits from the licenses it issues.

hydrochloric acid but dissolves readily in the strong acid, its behavior showing it to be a weaker base than arsanilic acid. On boiling its solution in sodium hydroxide ammonia is evolved. When rapidly heated in an open capillary tube it darkens and softens at 280°C., but does not melt.

0.1405 gm. of substance; 12.35 cc. of N (22.0°C., 761 mm.).
0.3205 gm. of substance; 0.1832 gm. of $Mg_2As_2O_7$.
Calculated for $C_8H_{11}O_4N_2As$: N, 10.22 per cent; As, 27.33 per cent.
Found: N, 10.18 per cent; As, 27.59 per cent.

Sodium Salt.—The pure acid is suspended in enough water to form a thick paste and carefully treated with 25 per cent sodium hydroxide solution until completely dissolved and the solution reacts neutral to litmus. Two volumes of alcohol are then added, the pure sodium salt quickly separating as thin, nacreous plates. After filtering and washing with 85 per cent alcohol it is air-dried and then contains one-half molecule of water of crystallization. The sodium salt is extremely soluble in cold water, the solution reacting neutral to litmus. The apparatus, filter paper, water, etc., used in the preparation of this salt must be free from calcium; otherwise a precipitate of the insoluble calcium salt will contaminate solutions of the sodium salt.

0.3921 gm. of air-dry substance, at 100°C. *in vacuo* over H_2SO_4; 0.0117 gm. loss.
Calculated for $C_8H_{10}O_4N_2AsNa.\ \frac{1}{2}\ H_2O$: H_2O, 2.95 per cent.
Found: H_2O, 2.98 per cent.
Anhydrous: 0.1503 gm. of substance; 12.45 cc. of N(25.0°C., 762 mm.).
0.2300 gm. of substance; 0.1195 gm. of $Mg_2As_2O_7$.
Calculated for $C_8H_{10}O_4N_2AsNa$: N, 9.46 per cent; As, 25.32 per cent.
Found: N, 9.52 per cent; As, 25.08 per cent.

The potassium and ammonium salts were prepared in the same way as the sodium salt and form thin, glistening, hexagonal, microscopic platelets. On adding a calcium chloride solution to a solution of the sodium salt the calcium salt gradually separates as microscopic wedge-shaped prisms, containing no water of crystallization. Magnesia mixture causes no precipitate in the cold, but on warming the magnesium salt separates as a microcrystalline powder. Heavy

metal salts give immediate precipitates, the silver salt forming aggregates of thin, microscopic needles.

N-Phenylglycineamide-*p*-arsonic acid was also prepared as follows from *N*-phenylglycine methyl ester-*p*-arsonic acid by the action of ammonia.

N-(Phenyl-4-Arsonic Acid)-Glycine Methyl Ester.—40 gm. of *N*-phenylglycine-*p*-arsonic acid[3] were treated with 120 gm. of dry methyl alcohol and 4 gm. of concentrated sulfuric acid. The mixture was boiled under a reflux condenser for 2 hours. The ester separated on cooling and scratching, the precipitation being completed by the addition of water. The filtered, washed, and dried product weighed 38 gm. It can be recrystallized from hot water or hot 95 per cent alcohol, separating from the former as microscopic needles and thin plates. It is very sparingly soluble in cold water, cold alcohol, or boiling acetone, and is fairly easily soluble in methyl alcohol, especially on warming. When rapidly heated it softens and darkens above 200°C. and decomposes at about 285°.

0.1560 gm. of substance; 7.0 cc. of N (21.0°C., 747 mm.).
0.3135 gm. of substance; 0.1665 gm. of $Mg_2As_2O_7$.
Calculated for $C_9H_{12}O_5NAs$: N, 4.84 per cent; As, 25.94 per cent.
Found: N, 5.12 per cent; As, 25.63 per cent.

The ester was converted into the amide as follows: 10 gm. of the ester were slowly added, with stirring, to 30 cc. of well chilled, concentrated ammonia. At first a thick paste of the ammonium salt of the ester was formed, but on allowing the mixture to rise to room temperature the reaction proceeded with formation of a clear solution. After 24 hours the excess of ammonia was removed, preferably *in vacuo*. On diluting with water, filtering, and acidifying with acetic acid, *N*-phenylglycineamide-*p*-arsonic acid separated in characteristic form. This was purified as described above and was identical in every way with the product obtained by the direct method. The yield was 80 per cent of the theory.

0.1297 gm. of substance; 11.5 cc. of N (19.5°C., 742 mm.).
Calculated for $C_8H_{11}O_4N_2As$: N, 10.22 per cent.
Found: N, 10.11 per cent.

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[3] German Patent, No. 204,664.

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CEPHALIN.

VII. The Glycerophosphoric Acid of Cephalin.

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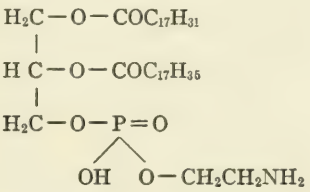
The generally accepted structural formula of cephalin is as follows:



This conception presumes the existence of glycerophosphoric acid in the molecule of cephalin. Thudichum,[1] who discovered cephalin, also was the first to make this assumption. Every other worker who followed Thudichum made similar assumptions. Parnas[2] was the first to call attention to the lack of evidence in favor of the theory, though admitting the possibility of its correctness. MacLean in his monograph on "Lecithin and allied substances" presents a very lucid summary of the weak points of the theory.

The main objection to the conclusions of the older writers lies in the fact that the material employed by them was not a pure cephalin but a mixture of this substance with lecithin.

In recent years, Fränkel and Dimitz[3] claimed to have identified glycerophosphoric acid as a component of cephalin. Unfortunately the evidence presented by these investigators is not convincing since they have failed to substantiate their claim with satisfactory analyt-

[1] Thudichum, J. L. W., The chemical constitution of the brain, London, 1884.

[2] Parnas, J., *Biochem. Z.*, 1909, xxii, 411.

[3] Fränkel, S., and Dimitz, L., *Biochem. Z.*, 1909, xxi, 337.

ical data. The analytical data are limited to one element; namely, barium.

Regardless of the meager proof of the nature of their substance, Fränkel and Dimitz have laid claim to a second very important discovery; namely, that the glycerophosphoric acid derived from cephalin was the optical isomer of the corresponding substance occurring in lecithin.

Willstätter and Lüdecke found[4] that the glycerophosphoric acid obtained from lecithin was optically active and levorotary. The substance obtained by Fränkel and Dimitz was dextrorotary, and hence followed the claim that it was isomeric to the substance described by Willstätter and Lüdecke.

The presence of glycerophosphoric acid in the cephalin molecule seemed *a priori* quite probable in the light of facts discovered in recent years. Simultaneously, Foster[5] and Levene and West[6] have shown that glycerol is present in cephalin in the quantity required by the generally accepted theory.

Furthermore, it was found that the basic part of cephalin is amino ethyl alcohol, and that in this base the alcoholic group only is substituted, the amino group remaining free.

Theoretically there are only two alternative ways in which glycerol may be attached to the phosphoric acid, either directly (forming glycerophosphoric acid) or indirectly. If the linking were indirect, then the glycerol would be attached to the aminoethanol. If that were so, then the amino group could not be free in the molecule of cephalin. Thus the presence of glycerophosphoric acid in cephalin could have been accepted even before the substance was actually isolated, although for the final proof, the isolation of the substance would have been desirable. On the other hand, without isolating the substance there was no way to reach a decision as to which of the possible isomers of glycerophosphoric acid is the one present in the molecule of cephalin. True, the optical rotation of hydrolecithin and of hydrocephalin is practically identical in direction and magni-

[4] Willstätter, R., and Lüdecke, K., *Ber. chem. Ges.*, 1904, xxxvii, 3753.

[5] Foster, M. L., *J. Biol. Chem.*, 1915, xx, 403.

[6] Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1916, xxiv, 50.

tude. Levene and West[7] have pointed out that this fact may be interpreted to signify that the glycerophosphoric acids in the two substances are identical. This conclusion contradicts the theory of Fränkel and Dimitz. Hence it became necessary to choose between the two views, and, in order to make the choice intelligently, an effort was made to isolate the glycerophosphoric acid of the cephalin molecule.

Glycerophosphoric Acid from Lecithin.

The present communication contains a report on the preparation of glycerophosphoric acid from both cephalin and lecithin. The latter was prepared for the following reasons. First, Willstätter and Lüdecke[4] stated that the magnitude of the optical rotation of the glycerophosphoric acid from lecithin differed depending on the mode of preparation. Second, the directions for the preparation of the substances given by these authors are so meager than any one repeating the work could have no assurance that he was following exactly the conditions of Willstätter and Lüdecke. Under such circumstances if the glycerophosphoric acid prepared by us from cephalin was found different from the one of Willstätter and Lüdecke, the difference could have been ascribed not to the inherent properties of the substance, but to the manner of its preparation.

The glycerophosphoric acid obtained by us from lecithin resembled the one obtained by Willstätter and Lüdecke, in respect to the direction of its rotation. It was levorotary. However, the magnitude of the rotation of our substance was lower than the maximum rotation found by the earlier workers. The differences are probably due to differences in handling, since all the samples were partially racemized.

The maximum rotation found by us was $[\alpha]_D^{20} = -0.74$, by the other writers $[\alpha]_D^{20} = -1.71$.

In a general way the crude substance was prepared in the manner indicated by Willstätter and Lüdecke. However, for purification it was found convenient to convert the barium salt into the lead salt, to purify the lead salt, and then to reconvert this into the barium salt.

As a rule, the hydrolysis was brought about at room temperature; however, for the sake of comparison one experiment was performed

[7] Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1918, xxxv, 287.

by boiling the hydrolysis mixture. This variation in the condition of hydrolysis remained without influence on the rotatory power of the resulting substance.

Hydrolysis of Cephalin.

After the most favorable conditions of hydrolysis of lecithin had been established, they were applied to the hydrolysis of cephalin. The result was practically identical. The glycerophosphoric acid obtained from cephalin, purified through conversion of the crude barium salt into the lead salt with subsequent reconversion of the latter into the barium salt, possessed the optical rotation of the same magnitude as the acid from lecithin; namely, $[\alpha]_D^{20} = -0.69$.

In this respect the result obtained by us is new. It establishes the fact that glycerophosphoric acid enters into the structure of cephalin, and further that the acid is identical with that present in lecithin. However, it is worthy of note that while the crude barium glycerophosphate obtained from lecithin always showed a levorotation, though of a comparatively low magnitude, the barium glycerophosphate from cephalin showed dextrorotation. The magnitude of this rotation was progressively descending on purification of the substance. All the dextrorotary samples were found to contain nitrogen, and the purification of the substance which led to the fall of the dextrorotation also led to the diminution in the nitrogen content. It is probable, therefore, that the claim of Fränkel and Dimitz to the discovery of a dextrorotary glycerophosphoric acid from cephalin was an error brought about by the fact that the substance analyzed by them was a mixture of glycerophosphoric acid with some product of intermediary hydrolysis.

EXPERIMENTAL.

Barium Glycerophosphoric Acid from Lecithin.

Hydrolysis at Low Temperature.

75 gm. of lecithin were obtained by the decomposition with ammonium carbonate of lecithin cadmium chloride containing 20 per cent amino nitrogen. This material was ground under water until a uni-

form emulsion was obtained, and then shaken for 6 hours at room temperature with 1 liter of a saturated aqueous solution of barium hydroxide (the equivalent of $2\frac{1}{4}$ mols of $Ba(OH)_2$). After standing for 3 hours, the mixture was filtered and the excess of barium contained in the filtrate removed quantitatively with sulfuric acid. The resulting filtrate was concentrated to a small bulk under diminished pressure, and the barium salt precipitated by pouring the thin syrup into several volumes of absolute alcohol. After one purification by dissolving in a small quantity of water, filtering through bone-black, and reprecipitating with alcohol, the yield of this material was 7.5 gm.

The barium salt obtained in this way was subjected to two more purifications by solution in water and precipitation by alcohol. Finally it was twice dissolved in a minimum amount of water and allowed to stand until it separated as a mass of heavy granules and noncrystalline scales. These were filtered with suction, washed with alcohol, and dried under diminished pressure. Obtained in this manner, the salt, when dry, is a light tan, granular material, which dissolves in water giving a clear yellow solution. Though very soluble in cold water it is not hygroscopic; on warming the cold solution a fine amorphous powder is precipitated which does not dissolve completely as the solution is cooled. It is insoluble in alcohol, ether, and acetone. All rotations were made in 20 per cent aqueous solution. A sample (No. 23) had the following composition. It contained no nitrogen.

0.1018 gm. of substance dried under diminished pressure at the temperature of zylene vapor gave on combustion 0.0472 gm. of CO_2, 0.0248 gm. of H_2O, and 0.0712 gm. of ash.

0.2000 gm. of substance containing 7.36 per cent moisture gave 0.0666 mg. of $Mg_2P_2O_7$.

0.1000 gm. of substance containing 7.36 per cent moisture gave 0.0668 mg. of $BaSO_4$.

	Calculated for $C_8H_7O_6PBa$. *per cent*	Found. No 23. *per cent*
C	11.71	12.64
H	2.29	2.72
P	10.10	10.02
Ba	44.68	42.43

The rotation of the substance was as follows:

$$[\alpha]_D^{20} = \frac{-0.075 \times 100}{1 \times 18.42} = -0.41.$$

As our investigation progressed, we found that the method of reprecipitation described above was not effective for the complete purification of the salts, as shown by their continued retention of nitrogen, and the process described below was applied to all our later products.

2 gm. of substance (No. 23) were dissolved in water. A 25 per cent aqueous solution of neutral lead acetate was added until precipitation was complete; the precipitate was filtered and thoroughly washed with water, alcohol, and ether. The lead salt thus isolated was suspended in water, and hydrogen sulfide passed through until the decomposition was complete. The lead sulfide was removed by filtration and the filtrate concentrated under diminished pressure to a small volume and made slightly alkaline to litmus with barium hydroxide. From the concentrated solution the barium salt was precipitated by adding several volumes of 95 per cent alcohol. The yield of this preparation, No. 63, was 1.5 gm.

When rapidly precipitated by alcohol, the barium salt separates in white amorphous flakes, which dry to a gleaming white powder. The color reported in the foregoing experiment is apparently due to an impurity as all samples obtained analytically pure were white when dry, and very readily soluble in water, giving colorless, water-clear solution. If the salt after filtration is not dried either by washing with alcohol and ether or by rapid desiccation, the amorphous flakes undergo an apparent fusion and dry to a mass of colorless granules and scales. This granular form can also be obtained by precipitating the salt from a very concentrated aqueous solution by a gradual addition of alcohol.

0.101 gm. of substance dried under diminished pressure at temperature of xylene vapor gave on combustion 0.0438 gm. of CO_2, 0.0194 gm. of H_2O, and 0.0742 gm. of ash.

0.2786 gm. of substance containing 9.11 per cent water gave 0.0912 gm. of $Mg_2P_2O_7$.

	Calculated for $C_3H_7O_6PBa$.	Found. No. 63.
	per cent	*per cent*
C	11.71	11.84
H	2.29	2.15
P	10.10	10.00
Ba	44.68	44.03

The rotation of No. 63 is

$$[\alpha]_D^{20} = \frac{-0.11 \times 100}{1 \times 18.82} = -0.58.$$

Both granular and flocculent forms after air-drying contain water of crystallization, the value of which is not constant, though in general it approaches $1\frac{3}{4}$ mols of H_2O. The nature of the substance, however, does not permit one to attribute too much importance to this value. After preliminary drying under diminished pressure over sulfuric acid at room temperature, the loss of water on complete drying was more constant, corresponding to $C_3H_7O_6PBa \cdot H_2O$.

After air-drying, on desiccation over sulfuric acid under diminished pressure at the temperature of xylene vapor, 0.1108 gm. of No. 63 lost 0.0098 gm. of H_2O.

	Calculated for $C_3H_7O_6PBa \cdot 1\frac{3}{4}H_2O$.	Found. No. 63.
	per cent	*per cent*
H_2O	9.30	8.84

The substance was dried for 24 to 48 hours under diminished pressure over sulfuric acid at room temperature. On further drying to constant weight at the temperature of xylene vapor, 0.1100 gm. of No. 56 lost 0.0060 gm. of H_2O and 0.1092 gm. of No. 57 lost 0.0058 gm. of H_2O.

	Calculated for $C_3H_7O_6PBa \cdot H_2O$.	Found.	
		No. 56.	No. 57.
	per cent	*per cent*	*per cent*
H_2O	5.53	5.45	5.31

In order to be certain of the source of the glycerophosphoric acid, lecithin cadmium chloride was isolated from egg oil by the method recommended by Levene and West[8] for the preparation of pure lecithin. The amino content of this material was negligible. From its cadmium salt the lecithin was isolated by treatment with $(NH_4)_2CO_3$ in boiling 85 per cent alcoholic solution.

[8] Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1918, xxxiv, 175.

75 gm. of this product were hydrolyzed with 1 liter of barium hydroxide solution, and the barium salt was isolated in the same manner as No. 23. 8 gm. of crude material were obtained from which the material most insoluble in alcohol was separated by repeated solution in water and fractional precipitation with small volumes of alcohol. After eighteen such reprecipitations, a substance (No. 55) of the following composition and analysis was obtained. It contained no nitrogen.

0.200 gm. of substance containing 5.03 per cent moisture gave 0.0706 gm. of $Mg_2P_2O_7$.

0.100 gm. of substance containing 5.03 per cent moisture gave 0.0690 gm. of $BaSO_4$.

	Calculated for $C_3H_7O_6PBa$. *per cent*	Found. No. 55. *per cent*
N	0.00	0.00
P	10.10	10.36
Ba	44.68	42.75

$$[\alpha]_D^{20} = \frac{-0.12 \times 100}{1 \times 18.89} = -0.63.$$

That a longer period of hydrolysis would give a better yield and not affect the rotation adversely seemed probable, and was proved by hydrolyzing 100 gm. of lecithin for 16 hours with 1,350 cc. of a saturated barium hydroxide solution. When isolated by the method described for No. 23 the yield of the crude material was 35 gm. (90 per cent of the theory). After three precipitations from alcohol this material (No. 56) had the following composition.

0.200 gm. of substance containing 5.45 per cent moisture gave 0.0572 gm. of $Mg_2P_2O_7$.

0.100 gm. of substance containing 5.45 per cent moisture gave 0.0664 gm. of $BaSO_4$.

0.200 gm. of substance containing 5.45 per cent moisture used for Kjeldahl nitrogen determination required 0.26 cc. of 0.1 N HCl.

	Calculated for $C_3H_7O_6PBa$. *per cent*	Found. No. 56. *per cent*
N	0.00	0.18
P	10.10	8.43
Ba	44.68	41.33

No. 56 was then further purified by precipitating the lead salt and decomposing this by the directions given above. The resulting substance (No. 57) had the following composition and rotation. It contained no nitrogen.

0.200 gm. of substance containing 5.31 per cent moisture gave 0.0704 gm. of $Mg_2P_2O_7$.

0.100 gm. of substance containing 5.31 per cent moisture gave 0.0680 gm. of $BaSO_4$.

	Calculated for $C_3H_7O_6PBa$. *per cent*	Found. No. 57. *per cent*
N	0.00	0.00
P	10.10	10.38
Ba	44.68	42.26

$$[\alpha]_D^{20} = \frac{-0.09 \times 100}{1 \times 19.36} = -0.46.$$

A second purification through the lead salt resulted in No. 60, whose rotation was

$$[\alpha]_D^{20} = \frac{-0.14 \times 100}{1 \times 18.92} = -0.74$$

A third purification by this method gave no further change in the rotation.

The final analysis after an additional precipitation with alcohol follows.

0.1026 gm. of substance dried *in vacuo* at temperature of xylene vapor gave on combustion 0.0482 gm. of CO_2, 0.0248 gm. of H_2O, and 0.0774 gm. of ash.

0.200 gm. of substance containing 7.06 per cent moisture gave 0.0696 gm. of $Mg_2P_2O_7$.

	Calculated for $C_3H_7O_6PBa$. *per cent*	Found. No. 78. *per cent*
C	11.71	12.81
H	2.29	2.70
P	10.10	10.43
Ba	44.68	44.87

Hydrolysis at Higher Temperature.

97 gm. of lecithin cadmium chloride were dissolved in 250 cc. of boiling 50 per cent alcohol and this solution was added to one containing 103 gm. ($3\frac{1}{4}$ mols) of barium hydroxide in 1 liter of hot water.

After 1 hour of gentle boiling the mixture was cooled and filtered. The excess of barium hydroxide was removed from the filtrate by quantitative precipitation with sulfuric acid, and the filtered solution concentrated in vacuum to small bulk. The addition of an equal volume of 95 per cent alcohol precipitated the salt of glycerophosphoric acid, but the barium chloride formed in the course of the hydrolysis remained in solution. Thorough washing with 50 per cent alcohol removed all traces of chlorides from the filtered barium glycerophosphoric acid. After two preliminary precipitations by alcohol, the salt was converted into the lead salt, decomposed, and again reprecipitated as the barium salt by the directions given above.

The specific rotation of this substance was

$$[\alpha]_D^{20} = \frac{-0.03 \times 100}{1 \times 18.89} = -0.16.$$

A second purification by conversion into the lead salt raised this rotation as follows:

$$[\alpha]_D^{20} = \frac{-0.12 \times 100}{1 \times 18.89} = -0.63.$$

On analysis 0.102 gm. (No. 69), dried under diminished pressure over sulfuric acid at temperature of xylene vapor, gave on combustion 0.0234 gm. of H_2O, 0.0449 gm. of CO_2, and 0.0724 gm. of ash.

0.300 gm. (No. 69), containing 7.77 per cent moisture, gave 0.1021 gm. of $Mg_2P_2O_7$.

	Calculated for $C_3H_7O_6PBa$. *per cent*	Found. No. 69. *per cent*
C	11.71	12.00
H	2.29	2.56
P	10.10	10.28
Ba	44.68	41.19

Barium Glycophosphoric Acid from Cephalin.

Preparation of Cephalin.

Three samples of cephalin were used as the source of the glycerophosphoric salts described below. All were obtained from ox brains by six extractions with ether containing 5 per cent water. This material, after evaporation of the ether, was precipitated with acetone and the fats and saturated phosphatides were removed from the pre-

cipitate by repeatedly dissolving in ether, removing all material insoluble in ether at 0°, and reprecipitating by pouring into acetone. From the mixture of lecithin and cephalin thus obtained, the cephalin was separated by precipitating the ethereal solution with alcohol. The first sample of cephalin was purified by repeated precipitation by alcohol from ethereal solution and finally three precipitations from a solution in gasoline (B. P. 50–60°). The analysis of this material (No. 44) is given below.

The second sample, after three precipitations from an ethereal solution by alcohol, was shaken with water until a uniform emulsion was obtained. From this the cephalin was precipitated by dilute hydrochloric acid, separated by centrifuging, and thoroughly washed with acetone. Further purification was effected by repeatedly dissolving either in ether (saturated with water at room temperature) or in gasoline (B. P. 50–60°) and precipitating with alcohol. This material (No. 58) had the composition indicated below.

The third sample was prepared in the following manner. The crude cephalin was exhaustively extracted with alcohol at room temperature and then repeatedly reprecipitated by alcohol from ether or gasoline solution, until its amino content was that indicated in the analysis (No. 135) recorded below. Following this the material was emulsified with water, precipitated by hydrochloric acid, and the precipitate washed with alcohol.

No. 44.

0.5 gm. was dissolved in 5 cc. of glacial acetic acid.

2 cc. of this solution for Kjeldahl determination required 340 cc. of 0.1 N HCl = 0.00468 gm. of N.

2 cc. of this solution for Van Slyke determination gave 8.55 cc. of N at $T°$ = 26°C. and P = 757.1 mm.; N = 0.00476 gm.

$$\text{Ratio } \frac{NH_2N}{\text{Total N}} = \frac{1}{1}$$

No. 58.

2 gm. of cephalin were hydrolyzed with HCl, neutralized, and concentrated to 25 cc.

5 cc. of this solution required for Kjeldahl determination 0.90 cc. of 0.1 N HCl = 0.00126 gm. of N.

2 cc. of this solution by Van Slyke determination gave 0.91 cc. of N at $T°$ = 24° and P = 755.9 mm.; N = 0.000505.

$$\text{Ratio } \frac{NH_2N}{\text{Total N}} = \frac{1}{1}$$

No. 135.

0.2 gm. was dissolved in 10 cc. of glacial acetic acid.

5 cc. of this solution for Kjeldahl determination required 1.27 cc. of 0.1 N HCl = 0.001778 gm. of N.

1 cc. of this solution for Van Slyke determination gave 0.65 cc. of N at $T°$ = 30° and P = 759.2 mm.; N = 0.003505 gm.

$$\text{Ratio } \frac{NH_2N}{\text{Total N}} = \frac{1}{1}$$

Hydrolysis of Cephalin.

75 gm. of cephalin (No. 44) were hydrolyzed by shaking for 9 hours at room temperature with a liter of saturated barium hydroxide solution. The directions given for No. 23 were followed in working up the resulting mixture. On pouring the concentrated aqueous solution into alcohol a colloidal solution formed which was precipitated by the addition of a few cc. of an aqueous solution of barium acetate. After thirteen reprecipitations by alcohol from its aqueous solution, this material (No. 51) had the following composition and rotation.

0.200 gm. of substance containing 5.67 per cent moisture gave 0.0556 gm. of $Mg_2P_2O_7$.

0.100 gm. of substance containing 5.67 per cent moisture gave 0.0662 gm. of $BaSO_4$.

0.200 gm. of substance used for Kjeldahl determination required 1.12 cc. of 0.1 N HCl.

	Calculated for $C_3H_7O_6PBa$. *per cent*	Found. No. 51. *per cent*
N	0.00	0.83
P	10.10	8.21
Ba	44.68	41.28

$$[\alpha]_D^{20} = \frac{+0.15 \times 100}{1 \times 18.88} = +0.80.$$

No. 51 five times reprecipitated by alcohol still retained nitrogen.

0.200 gm. of substance (No. 53) containing 9.28 per cent moisture required for Kjeldahl determination 0.77 cc. of 0.1 N HCl.

	Calculated for $C_3H_7O_6PBa$.	Found. No. 53.
	per cent	*per cent*
N	0.00	0.59

Its rotation was

$$[\alpha]_D^{20} = \frac{+0.08 \times 100}{1 \times 18.20} = +0.44.$$

Ten additional reprecipitations by alcohol gave a substance (No. 59) of the following composition and rotation.

0.200 gm. of substance containing 9.07 per cent moisture gave 0.0566 gm. of $Mg_2P_2O_7$.

0.100 gm. of substance containing 9.07 per cent moisture gave 0.0644 gm. of $BaSO_4$.

0.200 gm. of substance containing 9.07 per cent moisture used for Kjeldahl determination required 0.57 cc. of 0.1 N HCl.

	Calculated for $C_3H_7O_6PBa$.	Found. No. 59.
	per cent	*per cent*
N	0.00	0.40
P	10.10	8.67
Ba	44.68	41.68

$$[\alpha]_D^{20} = \frac{+0.04 \times 100}{1 \times 18.19} = +0.22.$$

All these salts agreed in property and appearance with the salts derived from the hydrolysis of lecithin by the same sort of purification. They all retained a distinctly yellow color which could not be wholly removed by bone-blacking the aqueous solution. All specimens were very soluble in water and rotations were made on 20 per cent aqueous solutions.

The mother liquors from the purifications of No. 51 and its derivatives were concentrated in vacuum to a small bulk and precipitated with alcohol. The bulky precipitate was filtered and again dissolved in water. To this was added a 25 per cent aqueous solution of neutral lead acetate, and on standing lead glycerophosphoric acid settled out. This salt was filtered, suspended in water, and decomposed by hydrogen sulfide. The precipitated lead sulfide was filtered and the filtrate concentrated to a small bulk. The barium salt of glycerophosphoric acid was again isolated by adding barium hydroxide until slightly

alkaline to litmus and precipitating with several volumes of alcohol. This material was a glistening white, amorphous powder, identical in all characteristics with No. 63 obtained in an analogous manner from lecithin. Its composition and rotation were as follows. It contained no nitrogen.

0.1594 gm. of substance containing 5.56 per cent moisture gave 0.0548 gm. of $Mg_2P_2O_7$.

0.103 gm. of substance containing 5.56 per cent moisture gave 0.0752 gm. of $BaSO_4$.

	Calculated for $C_6H_7O_6PBa$. *per cent*	Found. No. 64. *per cent*
P	10.10	10.14
Ba	44.68	45.48

$$[\alpha]_D^{20} = \frac{-0.13 \times 100}{1 \times 18.97} = -0.69.$$

Another hydrolysis of cephalin was made on 75 gm. of cephalin (No. 58) which were shaken for 16 hours at room temperature with $2\frac{1}{4}$ mols of barium hydroxide. The barium glycerophosphoric acid was isolated by the method used in the previous experiments, and after a preliminary precipitation with alcohol, was purified by conversion to the lead salt, and reprecipitation as the barium salt, according to the directions given under the foregoing experiment. After two additional precipitations by alcohol from its aqueous solution, the yield of this material (No. 68) was 5 gm. and its composition and rotation were the following.

0.101 gm. of substance dried under diminished pressure over sulfuric acid at temperature of xylene vapor yielded on combustion 0.0520 gm. of CO_2, 0.0218 gm. of H_2O, and 0.0684 gm. of ash.

0.200 gm. of substance containing 7.84 per cent moisture gave 0.0626 gm. of $Mg_2P_2O_7$.

	Calculated for $C_3H_7O_6PBa$. *per cent*	Found. No. 68. *per cent*
C	11.77	14.04
H	2.29	2.41
P	10.10	9.92
Ba	44.68	39.12

$$[\alpha]_D^{20} = \frac{-0.9 \times 100}{1 \times 18.40} = -0.49.$$

A second purification through the lead salt and one reprecipitation from alcohol gave a substance (No. 77) of the following composition and rotation.

0.099 gm. of substance dried under diminished pressure over sulfuric acid in xylene bath gave on combustion 0.0484 gm. of Mg_2CO_2, 0.0222 gm. of H_2O, and 0.0692 gm. of ash.

0.300 gm. of substance containing 9.00 per cent moisture gave 0.0956 gm. of $Mg_2P_2O_7$.

	Calculated for $C_3H_7O_6PBa$. *per cent*	Found. No. 77. *per cent*
C	11.71	13.33
H	2.29	2.50
P	10.10	9.76
Ba	44.68	41.36

$$[\alpha]_D^{20} = \frac{-0.12 \times 100}{1 \times 18.00} = -0.67.$$

A third hydrolysis of cephalin was carried out in the same way on 50 gm. of cephalin (No. 135) and the purification of this material was exactly like that giving rise to No. 77. The analysis and rotation of this material were as follows:

0.1011 gm. of substance dried under diminished pressure over sulfuric acid in xylene bath, gave on combustion 0.044 gm. of CO_2, 0.0216 gm. of H_2O, and 0.0724 gm. of ash.

0.200 gm. of substance containing 8.80 per cent moisture gave 0.0648 gm. of $Mg_2P_2O_7$.

	Calculated for $C_3H_7O_6PBa$. *per cent*	Found. No. 145. *per cent*
C	11.71	11.86
H	2.29	2.40
P	10.10	9.89
Ba	44.68	43.88

$$[\alpha]_D^{20} = \frac{-0.13 \times 100}{1 \times 20.05} = -0.65.$$

Like the barium glycerophosphates obtained from lecithin, these salts, after air-drying, contained variable amounts of water of crystallization approaching $1\frac{3}{4}$ mols; while after desiccation under diminished pressure for about 24 hours at room temperature, a more constant content of water was retained, corresponding to $C_3H_7O_6PBa \cdot H_2O$.

On complete desiccation over H_2SO_4 under diminished pressure at the temperature of xylene vapor, the following results were obtained.

0.1256 gm. of No. 59, previously air-dried, lost 0.0114 gm. of H_2O.
0.1088 " " " 77, " " " 0.0098 " " H_2O.
0.1104 " " " 78, " " " 0.0093 " " H_2O.

	Calculated for $C_3H_7O_6PBa \cdot 1\frac{1}{2}H_2O$.	Found. No. 59.	No. 77.	No. 78.
	per cent	*per cent*	*per cent*	*per cent*
H_2O	9.30	9.07	9.00	9.29

The substance dried at room temperature under diminished pressure lost on drying to constant weight at the temperature of xylene vapor as follows:

0.1164 gm. of No. 51 lost 0.0066 gm. of H_2O.
0.1116 " " " 64 " 0.0062 " " H_2O.

	Calculated for $C_3H_7O_6PBa \cdot H_2O$.	Found. No. 51.	No. 64.
	per cent	*per cent*	*per cent*
H_2O	5.53	5.67	5.56

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CRYSTALLINE GUANYLIC ACID.

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(From the Laboratories of The Rockefeller Institute for Medical Research.)

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The theory of the polynucleotide structure of the yeast nucleic acid has been receiving confirmation in recent years through the investigations of several workers. Levene,[1] Jones and Kennedy,[2] and Thannhauser and Dorfmüller[3] have described crystalline mononucleotides obtained from yeast nucleic acid. The present communication contains a report on guanylic acid obtained in crystalline form. It crystallized in the form of long prismatic needles having the same appearance as guanosine. The substance had all the properties of guanylic acid. It gelatinized in the presence of mineral impurities. A test for the presence of free phosphoric acid was negative. It had no melting point but turned brown at 208°C. The optical rotation in water solution was $[\alpha]_D^{20} = -7.5$, and in a 5 per cent ammoniacal solution $[\alpha]_D^{20} = -43.5$. On hydrolysis it gave guanosine or guanine sulfate depending on the conditions of the experiment. The free acid, when air-dry, crystallized with 2 mols of crystal water, and, when dried under diminished pressure at the temperature of a toluene vapor bath to constant weight, still retained $\frac{1}{2}$ mol of crystal water. The pure guanylic acid was converted into a crystalline brucine salt.

EXPERIMENTAL.

Crude brucine salt of guanylic acid was converted into the ammonium salt in the manner described in a previous publication.[4] The ammonium salt was dissolved in hot water. The resulting solution

[1] Levene, P. A., *Proc. Soc. Exp. Biol. and Med.*, 1917–18, xv, 21.

[2] Jones, W., and Kennedy, R. P., *J. Pharmacol. and Exp. Therap.*, 1919, xii, 253.

[3] Thannhauser, S. J., and Dorfmüller, G., *Z. physiol. Chem.*, 1919, civ, 65.

[4] Levene, P. A., *J. Biol. Chem.*, 1919, xxxix, 77.

was acidulated, and the substance precipitated by means of neutral lead acetate. The precipitate was repeatedly washed with water, then suspended in water, and freed from lead. The resulting solution was again precipitated with neutral lead, and the process repeated once more. Finally the solution obtained on decomposition of the lead salt gave on concentration under diminished pressure a crystalline deposit.

On drying under diminished pressure at the temperature of xylene vapor, the substance still retained $\frac{1}{2}$ mol of crystal water.

0.1084 gm. of the vacuum-dry substance gave 0.1274 gm. of CO_2 and 0.0424 gm. of H_2O.

0.1834 gm. of the substance used for Kjeldahl nitrogen estimation required 24.45 cc. of 0.1 N acid for neutralization.

0.2757 gm. of the substance gave 0.0844 gm. of $Mg_2P_2O_7$.

	Calculated for $C_{10}H_{14}N_5O_8+\frac{1}{2}H_2O$.	Found.
	per cent	*per cent*
C	32.24	32.05
H	4.03	4.38
N	18.80	18.67
P	8.33	8.55

In 10 per cent hydrochloric acid the substance was optically inactive. In aqueous solution the rotation was

$$[\alpha]_D^{20} = \frac{-0.15 \times 100}{1 \times 2} = -7.5$$

In 5 per cent aqueous ammonia solution the rotation was

$$[\alpha]_D^{20} = \frac{-0.87 \times 100}{1 \times 2} = -43.5$$

Acid Hydrolysis of the Substance.

2.0 gm. of the substance were dissolved in 20.0 cc. of 2 per cent sulfuric acid and boiled over a flame with a return condenser for 1 hour. The substance was filtered and allowed to cool. Soon a crystalline deposit formed. This was filtered and recrystallized out of 5 per cent sulfuric acid. The substance was dried over night over sulfuric acid in a vacuum desiccator.

0.0905 gm. of dry substance gave 27.6 cc. of nitrogen gas at $T° = 25°C.$ and $P = 763$.

	Calculated for $(C_5H_5N_5O)_2H_2SO_4$.	Found.
	per cent	*per cent*
N.....	35.00	35.08

Ammonia Hydrolysis.

2.5 gm. of the substance were dissolved in 50 cc. of water containing 5 cc. of ammonia water, and the solution was made up to 75 cc. It was then heated in a sealed tube for 4 hours at 135°C. On cooling, the contents of the tube practically solidified. The solid material was filtered on suction and the residue recrystallized twice out of water. The crystals had the characteristic appearance of guanosine. The yield of the recrystallized substance was 0.6 gm.

0.1000 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 8.8 cc. of 0.1 N acid.

	Calculated for $C_{10}H_{13}N_5O_5 \cdot 2H_2O$.	Found.
	per cent	*per cent*
N.....	21.94	21.98

The rotation of the substance was

$$[\alpha]_D^{20} = \frac{-0.66 \times 100}{1 \times 1} = -66.0$$

Since this rotation was slightly higher than the one previously recorded (− 60.5), and since in the earlier work a less sensitive instrument was employed for the measurement, the rotation of a pure sample of guanosine was measured under exactly the same conditions as the sample obtained in the present work. The value obtained was

$$[\alpha]_D^{20} = \frac{-0.66 \times 100}{1 \times 1} = -66.0$$

Brucine Salt.

2 gm. of the crystalline guanylic acid were dissolved in water and neutralized by means of a solution of brucine in methyl alcohol. A crystalline deposit formed immediately. This was filtered off and

recrystallized out of a 35 per cent solution of alcohol in water. The air-dry substance contracted at 217° (Anschutz thermometer), melted into a brown liquid at 233°, and effervesced at 240°C.

0.1000 gm. of the substance gave on combustion 0.1938 gm. of CO_2 and 0.0558 gm. of H_2O.

0.2000 gm. of the substance gave 18.2 cc. of nitrogen gas at $T° = 25°$ and $P = 759$.

0.300 gm. of the substance gave 0.262 gm. of $Mg_2P_2O_7$.

	Calculated for $C_{56}H_{64}N_9O_{16}\cdot 7H_2O$.	Found.
	per cent	*per cent*
C	52.61	52.85
H	6.78	6.24
N	9.88	10.41
P	2.43	2.43

The rotation of the substance in 35 per cent alcohol solution was

$$[\alpha]_D^{20} = \frac{-0.52 \times 100}{1 \times 2} = -26.0$$

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SYNTHESES IN THE CINCHONA SERIES. II.

Quaternary Salts.

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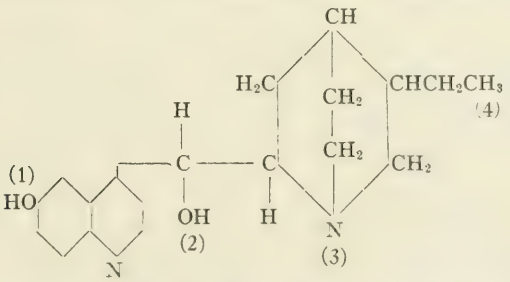
Received October 1, 1919.

In a recent communication a plan was outlined for the synthesis of aromatic arsenic compounds for biological study.[1] The present paper and that following it represent an attempt to apply a similar method of treatment to the field of the cinchona alkaloids, whose possibilities as material for chemotherapeutic study in one direction have been shown by the remarkable specificity for the penumococcus displayed by ethylhydrocupreine.[2]

Without entering into any extended discussion it may be stated that previous synthetic studies in the cinchona group have consisted mainly in the preparation of ethers of the phenolic hydroxyl group at (1) in the formula (ethylhydrocupreine being the ethyl ether), and in the formation of a large variety of esters, making use of the alcoholic hydroxyl group (2), many of the latter substances having been produced in the search for a tasteless quinine derivative.



Several years ago the writers made an extended study of the quaternary salts of hexamethylene-tetramine, many of which proved to

[1] This Journal, **41**, 1581 (1919).

[2] Morgenroth and Levy, *Berl. klin. Wochschr.*, **48**, 1560, 1979 (1911).

possess considerable bactericidal power.[1] Since the cinchona alkaloids also contain a reactive tertiary amine group at (3) it was thought possible that from these also bactericidal, or more specifically, pneumococcidal substances might be synthesized using this as a basis. The present paper gives the chemical part of the work, and the bacteriological and biological findings will be reported in the appropriate place by Dr. Martha Wollstein, who has had charge of this phase of the work.

The methochlorides are, of course, the simplest quaternary salts possible, and a number of these were prepared as a basis for comparison. Next in complexity come the benzyl chlorides, several of which were obtained crystalline for the first time. Another larger, new group consisted of the salts formed with the chloroacetyl derivatives of ammonia, the lower aliphatic amines, and of benzylamine. Up to this point the salts were in general fairly readily soluble in water and in dilute solution did not give precipitates with alkali. In the case of the chloroacetyl derivatives of the aromatic amines the new series of salts with the alkaloids were only sparingly soluble in cold water, and were, therefore, converted into their water-soluble hydrochlorides in order to render them suitable for biological testing. The aqueous solutions so obtained gave precipitates of the quaternary bases on making alkaline with sodium hydroxide, but in only one case was a crystalline base obtained. The quaternary salts with the aromatic chloroacetyl derivatives also dissolved in dil. nitric acid, most of them soon depositing beautifully crystalline, sparingly soluble dinitrates or chloride nitrates, a representative of each group being described and analyzed. Most of the salts prepared crystallized with water of crystallization, or in cases in which anhydrous solvents were used, came to final equilibrium in the air with water of crystallization. Solutions of all of the salts, aliphatic as well as aromatic, yielded immediate precipitates with solutions of picric acid or potassium dichromate. The salts of alkaloids in which the side-chain at (4) had been hydrogenated and with halides containing no readily oxidizable group, also gave purple precipitates with potassium permanganate.

[1] *J. Biol. Chem.*, **20**, 659, 685 (1915); **21**, 103, *et seq.* (1915); *J. Exp. Med.*, **23**, 563, *et seq.* (1916).

EXPERIMENTAL.

The quaternary salts of the alkaloids with the chloroacetyl derivatives of the aliphatic and aromatic amines were prepared by boiling equimolecular amounts of the components in dry acetone for 5 to 7 hours, and this method is to be assumed below unless another is given. Unfortunately, no general method can be given for the isolation and purification of the salts, for although many either crystallized directly from the reaction mixture or remained as a crystalline residue when this was evaporated, most of the salts required different treatment in each case. Water of crystallization was determined in all cases after allowing the salt to come to equilibrium in the the air; water was then driven off *in vacuo* over sulfuric acid at room temperature, 80°, or 100°, depending on the properties of the salt. In the case of the hydrochlorides of the quaternary salts, most of those which were recrystallized from an aqueous solvent were first air-dried; all were dried for analysis to constant weight *in vacuo* at room temperature over sulfuric acid and crushed alkali. Nitrogen was usually determined by the Dumas method, although accurate values were also obtained by proper use of the Kjeldahl method. Halogen was determined in the majority of cases by the Volhard method, and it was sometimes necessary, owing to the formation of difficultly soluble nitrates which occasionally separated as gums before crystallizing, to add a definite excess of the silver nitrate solution and heat on the water bath before proceeding with the analysis. Optical rotations were calculated by means of the formula $[\alpha] = \alpha \times 100/l \times c$, taking c as g. of substance per 100 cc. solvent, a close approximation for low concentrations. Melting points above 140° were corrected to the short-stem thermometer basis.

(*A*) *Quaternary Salts of Cinchonine.*

Cinchonine Methochloride.—Although this salt is mentioned in the literature[1] it does not seem to have been described. 16.8 g. of cinchonine methiodide were suspended in water and shaken for two hours with freshly precipitated silver chloride. The mixture was warmed

[1] Stahlschmidt, *Ann.*, **90**, 219 (1854); Rosenstein, *Compt. rend.*, **130**, 753 (1900).

on the water bath, filtered hot, and the filtrate treated with sodium chloride, causing the gradual deposition of the salt, which was recrystallized from water, forming rhombic crystals containing one molecule of water of crystallization. The yield was 9 g. When rapidly heated to 265°, then slowly, the air-dry or anhydrous chloride decomposes at 270°, with preliminary darkening. It dissolves readily in methyl or ethyl alcohol, less easily in water, and is almost insoluble in acetone. In water $[\alpha]_D^{20.5}$ is + 225.1°, c = 1.482. The taste is weakly bitter.

Subs. (air-dry), 0.6863: loss, 0.0344 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{20}H_{25}ON_2Cl.H_2O$: H_2O, 4.97. Found: 5.01.
Subs. (anhydrous), 0.2432:AgCl, 0.1012.
Calc. for $C_{20}H_{25}ON_2Cl$: Cl, 10.28. Found: 10.29.

Cinchonine Benzyl Chloride.—Equimolecular amounts of cinchonine and benzyl chloride were boiled daily on the water bath with acetone for about two weeks. Although considerable cinchonine remained unattacked this method avoided the partial decomposition occurring when alcohol is used.[1] The mixture was finally chilled, filtered, and the precipitate boiled with water, filtering hot. The chloride crystallized on cooling as minute rods containing no water of crystallization and melting and decomposing to a purple mass at about 280°, with preliminary darkening. Claus and Treupel give the decomposition point as 248°. The salt dissolves in hot water or alcohol, sparingly in hot acetone or chloroform, or in the cold in water or alcohol. $[\alpha]_D^{22}$ in water = + 164.8°, c = 0.716.

Subs., 0.2563: AgCl, 0.0870.
Calc. for $C_{26}H_{29}ON_2Cl$: Cl, 8.43. Found: 8.40.

Cinchonine Iodoacetamide, $C_{19}H_{22}ON_2.ICH_2CONH_2$.—15 g. of cinchonine were dissolved in boiling alcohol and treated with 9.3 g. of iodoacetamide. After letting cool the solution was partially concentrated *in vacuo*, let stand overnight, and filtered from the precipitate of unchanged cinchonine. The filtrate, concentrated almost to dryness on the water bath, crystallized on rubbing with water, yielding 16 g. of the crude iodide. Recrystallized from water with the aid of boneblack it forms aggregates of long, thin plates and flat, prismatic

[1] Claus and Treupel, *Ber.*, **13**, 2294 (1880).

needles containing 2 molecules of water of crystallization. The anhydrous salt gradually softens above 145° to a yellow jelly which darkens as the temperature is raised and decomposes slowly at about 190°. It is very easily soluble in methyl or ethyl alcohol and dissolves readily in hot water and sparingly in the cold.

Subs. (air-dry), 0.6344: loss, 0.0414 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{21}H_{26}O_2N_3I.2H_2O$: H_2O, 6.99. Found: 6.53.
Subs. (anhydrous), 0.1403: 10.6 cc. N. (29.0°, 761 mm.). Subs., 0.1641: AgI, 0.0778.
Calc. for $C_{21}H_{26}O_2N_3I$: N, 8.77; I, 26.48. Found: N, 8.56; I, 25.62.

Cinchonine Chloroacetamide.—14.5 g. of cinchonine iodoacetamide were converted into the chloro compound and this isolated exactly as in the case of the methochloride. The product was recrystallized from a small volume of water, separating as rosets of minute, narrow platelets containing two molecules of water of crystallization, and melting with effervescence at 110–20° when rapidly heated. The yield was 9 g. The anhydrous chloride gradually melts to a yellow jelly when heated and liquefies completely at about 195°, with darkening and slow gas evolution. $[\alpha]_D^{25}$ in water is + 174.2°, c = 1.163. It dissolves readily in water, methyl or ethyl alcohol, or chloroform, and softens under dry acetone, dissolving with difficulty. The air-dry chloride dissolves rather sparingly in cold water, a suspension clearing on adding sodium hydroxide.

Subs. (air-dry), 0.8162: loss, 0.0698 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{21}H_{26}O_2N_3Cl.2H_2O$: H_2O, 8.51. Found: 8.55.
Subs. (anhydrous), 0.1513: 14.3 cc. N (23.0°, 753 mm.).
Subs., 0.1784: 9.01 cc. $AgNO_3$ soln. (1 cc. = 0.001812 g. Cl).
Calc. for $C_{21}H_{26}O_2N_3Cl$: N, 10.84; Cl, 9.15. Found: N, 10.80; Cl, 9.15.

An attempt was made to prepare the substance directly from cinchonine and chloroacetamide in boiling acetone but little ionized halogen was formed.

(*B*) *Quaternary Salts of Cinchonidine.*

Cinchonidine Methochloride.—This substance was prepared and isolated in the same way as its cinchonine stereoisomer, except that it was necessary to saturate the solution with salt after removal of the

silver iodide. The crude product was sucked as dry as possible, dissolved in absolute alcohol, filtered from sodium chloride, and cautiously precipitated by the addition of dry ether. A repetition of the purification process proved to be necessary. The salt forms minute needles containing between one and 1.5 molecules of water of crystallization. The test for alcohol of crystallization was negative. When rapidly heated to 230°, then slowly, the anhydrous compound melts and decomposes at 232–3° with slight preliminary softening. $[\alpha]_D^{22}$ in water is $-142.7°$, $c = 1.489$. The air-dry salt begins to melt about 20° lower than the dried portion. Claus and Bock[1] give 158° as the melting point of the anhydrous salt. It dissolves readily in the cold in water, methyl or ethyl alcohol, or chloroform, and only sparingly in dry acetone.

Subs. (air-dry), 0.6458: loss, 0.0394 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{20}H_{25}ON_2Cl.H_2O$: H_2O, 7.27. Found: 6.10.
Subs. (anhydrous), 0.2441: AgCl, 0.1031.
Calc. for $C_{20}H_{25}ON_2Cl$: Cl, 10.28. Found: 10.44.

Cinchonidine Chloroacetamide, $C_{19}H_{22}ON_2.ClCH_2CONH_2$.—15 g. of cinchonidine were dissolved in warm chloroform and treated with 9.5 g. of iodoacetamide, warming until clear. The next day about one-half of the chloroform was evaporated off and the iodide precipitated by means of dry ether. The crude product showed no tendency to crystallize and was accordingly dissolved in hot alcohol, diluted with water until just faintly turbid, and converted into the chloride with freshly precipitated silver chloride. The filtrate was concentrated to dryness *in vacuo*, evaporated down twice with absolute alcohol *in vacuo*, and finally taken up in absolute alcohol and fractionally precipitated with dry ether. The first gummy portions were discarded until finally a white, solid, amorphous product was precipitated. This was filtered off, washed with dry ether, and dried, the yield being 3.5 g. When heated it gradually softens and turns yellow, becoming completely fluid at about 190°. $[\alpha]_D^{22.5}$ in water is $-112.5°$, $c = 1.422$. The salt dissolves readily in water, methyl or ethyl alcohol, or chloroform, and forms a gum under dry acetone, dissolving with difficulty.

[1] *Ber.*, **13**, 2191 (1880).

Subs. 0.1416: 13.3 cc. N (24.5°, 755 mm.).
Subs. 0.1720: 8.59 cc. $AgNO_3$ soln. (1 cc. = 0.001812 g. Cl).
Calc. for $C_{21}H_{26}O_2N_3Cl$: N, 10.84; Cl, 9.15. Found: N, 10.72; Cl, 9.05.

(C) *Quaternary Salts of Quinine.*

Quinine Methochloride.—20.5 g. of quinine methiodide were converted into the chloride in the usual manner, salting out the compound by means of sodium chloride solution. After recrystallization from absolute alcohol, 11.6 g. of the methochloride were obtained as delicate needles containing two molecules of water of crystallization. A test for alcohol of crystallization was negative. The anhydrous salt melts partially at 170–5°, liquefying completely at 196–8° to an orange fluid filled with bubbles. $[\alpha]_D^{20} = -211.7°$ in water, $c = 0.655$. The salt is sparingly soluble in cold water, readily on heating, and dissolves freely in alcohol and very easily in methyl alcohol. Claus and Mallmann[1] describe the methochloride as needles containing one molecule of water of crystallization, melting at 181–2° when dry, and dissolving readily in water.

Subs. (air-dry), 0.7396: loss, 0.0642 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{21}H_{27}O_2N_2Cl.2H_2O$: H_2O, 8.77. Found: 8.68.
Subs. (anhydrous), 0.2578: AgCl, 0.0986.
Calc. for $C_{21}H_{27}O_2N_2Cl$: Cl, 9.46. Found: 9.46.

Quinine Benzyl Chloride.—17 g. of quinine were dissolved in warm acetone and allowed to stand in a warm place with 6.3 g. of benzyl chloride, adding acetone from time to time as it evaporated. After about a week a test portion cleared completely on dilution with water and addition of acid. Most of the acetone was then boiled off and absolute alcohol added. On warming and adding several volumes of dry ether crystallization of the salt commenced. The yield was 19.1 g. Recrystallized from absolute alcohol the chloride forms faintly yellow, rhombic prisms containing one molecule of water of crystallization. A test for alcohol of crystallization was negative. When rapidly heated to 180°, then slowly, the air-dry or anhydrous salt melts and darkens at 183–8°. $[\alpha]_D^{22}$ of the anhydrous compound in water is $-230.5°$, $c = 1.479$. The chloride has a bitter taste and

[1] *Ber.*, **14**, 76 (1881).

dissolves freely in cold water, methyl or ethyl alcohol, or acetone; less easily in ethyl acetate, and sparingly in chloroform. Mazzara and Possetto,[1] who prepared the salt in alcohol solution, describe it as amorphous.

Subs. (air-dry), 0.8335: loss, 0.0295 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{27}H_{31}O_2N_2Cl.H_2O$: H_2O, 3.84. Found: 3.54.
Subs. (anhydrous), 0.1311: 7.4 cc. N (23.5°, 749 mm.). Subs., 0.2668: AgCl, 0.0844.
Calc. for $C_{27}H_{31}O_2N_2Cl$: N, 6.22; Cl, 7.87. Found: N, 6.40; Cl, 7.83.

Quinine Iodoacetamide.—After about 1.5 hours an acetone solution of 16.2 g. of quinine and 9.3 g. of iodoacetamide at room temperature began to deposit the quaternary salt as rhombic crystals, 22.8 g. being obtained. Recrystallized from water it first softens to a paste, and separates as prisms containing 1.5 molecules of water of crystallization. In this form the iodide dissolves readily in methyl or ethyl alcohol or acetone, and is sparingly soluble in cold water, readily in hot. The anhydrous salt gradually darkens and forms a jelly when heated, finally melting completely at 175–80°. It dissolves readily in chloroform and shows $[\alpha]_D^{23} = -152.2°$ in water, $c = 1.009$.

Subs. (air-dry), 0.4789: loss, 0.0257 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{22}H_{28}O_3N_3I.1.5H_2O$: H_2O, 5.04. Found: 5.37.
Subs. (anhydrous), 0.1962: AgI, 0.0898.
Calc. for $C_{22}H_{28}O_3N_3I$: I, 24.93. Found: 24.73.

Quinine Chloroacetamide.—20 g. of the iodide were shaken in water for two hours with excess silver chloride, warmed, and filtered. The filtrate was concentrated to dryness *in vacuo*, taken up twice in absolute alcohol and concentrated as before, and finally dissolved in absolute alcohol and cautiously precipitated by the addition of dry ether. The solid, amorphous precipitate was collected, ground up under dry ether, filtered again, and dried, the yield being 11.8 g. The chloride gradually softens above 160° and appears completely fluid at 190°, with gas evolution. $[\alpha]_D^{25}$ in water is $-159.6°$, $c = 1.473$. The salt dissolves readily in water, methyl or ethyl alcohol, or chloroform, and only sparingly in acetone. On boiling an aqueous solution with sodium hydroxide ammonia is evolved.

[1] *Gazz. chim. ital.*, **13**, 530 (1884).

Subs., 0.1387: 12.45 cc. N (24.5°, 768 mm.). Subs., 0.2321: AgCl, 0.0787.
Calc. for $C_{22}H_{28}O_3N_3Cl$: N, 10.06; Cl, 8.49. Found: N, 10.41; Cl, 8.39.

Quinine Chloroacetanilide (*Quinine Acetanilide Chloride*), $C_{20}H_{24}O_2N_2.ClCH_2CONHC_6H_5$.—The method given at the beginning of the "Experimental Part" was used, with 8.5 g. of chloroacetanilide.[1] After boiling off the acetone dry chloroform was added to the already partially crystalline residue. After letting stand 19 g. of silky needles of the quaternary salt were obtained. It was purified by dissolving in absolute alcohol, filtering with the aid of boneblack, and adding several volumes of dry ether, separating after rubbing or seeding as rosets of delicate needles, which are quite hygroscopic until dried. When rapidly heated to 220°, then slowly, the salt darkens slightly above 190° and melts with decomposition at 224–5°. $[\alpha]_D^{23.5}$ in absolute alcohol is $-131.5°$, $c = 1.046$. It dissolves readily in methyl or ethyl alcohol, sparingly in dry acetone. It dissolves in dry chloroform, quickly separating again apparently with chloroform of crystallization. It gelatinizes under cold water and dissolves on heating, separating again on cooling as a vaseline-like mass. It dissolves with effervescence in conc. sulfuric acid, giving a pale yellow-green, fluorescent solution. The compound also gives the thalleoquinine test.

Subs., 0.1916: 14.35 cc. N (19.0°, 760 mm.).
Subs., 0.1731: 6.92 cc. $AgNO_3$ soln. (1 cc. = 0.001812 g. Cl).
Calc. for $C_{28}H_{32}O_3N_3Cl$: N, 8.51; Cl, 7.18. Found: N, 8.57; Cl, 7.24.

The Hydrochloride.—Quinine chloroacetanilide in absolute alcohol was treated with alcoholic hydrochloric acid, the hydrochloride separating quickly as aggregates of minute, flat needles, and was filtered off after adding an equal volume of dry ether. The crude salt was dissolved in hot absolute alcohol, crystallized by the cautious addition of dry ether, and dried *in vacuo* at room temperature over sulfuric acid and crushed alkali. When rapidly heated to 200°, then slowly, it darkens and melts with decomposition at 204–6° to a deep red mass.

It has a sharp, bitter taste, and dissolves readily in water, yielding a solution acid to litmus but not to congo red. It dissolves very readily in methyl alcohol, less easily in absolute alcohol, or dry chloroform, and is very difficultly soluble in dry acetone. An aqueous solution gives an amorphous precipitate with excess alkali.

[1] THIS JOURNAL, 39, 1441 (1917).

Subs., 0.1881: 13.1 cc. N (24.5°, 758 mm.).
Subs., 0.1258: 9.05 cc. $AgNO_3$ soln. (1 cc. = 0.001812 g. Cl).
Calc. for $C_{28}H_{32}O_3N_3Cl.HCl$: N, 7.93; Cl, 13.37. Found: N, 7.97; Cl, 13.04.

Quinine Chloroacetanilide Nitrate, $C_{20}H_{24}O_2N_2.ClCH_2CONHC_6H_5.HNO_3$.—By analogy with the corresponding derivative of hydroquinine (see below) this salt was originally believed to be quinine acetanilide dinitrate, but as it persistently retained several per cent. of chlorine in spite of attempts to substitute the nitrate ion the preparation was modified as follows: 2 g. of the chloride hydrochloride were dissolved in water with the aid of a drop of dil. nitric acid to drive back dissociation.

A few drops of saturated salt solution were added and then 10% nitric acid until the initial turbidity barely redissolved and crystals began to form. The salt separated rapidly on rubbing and after letting stand in the cold it was filtered off and dissolved in hot water. The solution was then cooled to initial turbidity, cleared with a drop of dil. nitric acid and treated with a few drops of saturated sodium chloride solution, then with 10% nitric acid until crystals began to separate. After finally letting stand in the ice box the deposit of thin, nacreous platelets was filtered off and washed with a little ice water. The yield was 1.4 g. When rapidly heated to 100°, then slowly, the salt decomposes at 201–3° with preliminary browning and softening. It is appreciably soluble in water at room temperature, but is almost insoluble in the presence of nitric acid. It is readily soluble in boiling water, less easily in boiling methyl or ethyl alcohol, and difficultly in boiling acetone or chloroform. The air-dry salt contains no water of crystallization.

Subs., 0.1163: 10.9 cc. N (28.5°, 763 mm.).
Subs., 0.1352: 4.54 cc. $AgNO_3$ soln. (1 cc. = 0.001794 g. Cl).
Calc. for $C_{28}H_{32}O_3N_3Cl.HNO_3$: N, 10.06; Cl, 6.37. Found: N, 10.65; Cl, 6.03.

Quinine p-Chloroacetylamino-phenol, $C_{20}H_{24}O_2N_2.ClCH_2CONHC_6H_4OH(p\text{-})$.—4.7 g. of *p*-chloroacetylamino-phenol[1] were used. Toward the end a dark tar separated, and on standing overnight a portion of this crystallized. The acetone was poured off and the crude material dissolved in a little warm absolute alcohol and treated with several

[1] This Journal, 39, 1442 (1917).

volumes of dry acetone. Dry, alcohol-free ether was then cautiously added until a slight permanent turbidity had formed, and after treating with boneblack, the filtrate was seeded. On rubbing and stirring and occasionally adding additional small amounts of dry ether, 5.1 g. of the chloride separated as rosets of minute, faintly pinkish needles. The salt was washed with dry acetone, dried *in vacuo* over sulfuric acid to remove adhering solvent, and then air-dried, coming to equilibrium with approximately 1.5 molecules of water of crystallization. It then dissolves with difficulty in cold water, freely on heating, separating on cooling as a jelly. An aqueous solution gives a precipitate with sodium hydroxide. The anhydrous salt softens at about 180–90° to an orange-red jelly which is completely fluid at about 205° and slowly evolves gas. It dissolves readily in methyl or ethyl alcohol, forms a gum under dry chloroform, and is sparingly soluble in cold, dry acetone, more easily on warming.

Subs. (air-dry), 0.4644: loss, 0.0258 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{28}H_{32}O_4N_3Cl.1.5H_2O$: H_2O, 5.03. Found: 5.56.
Subs. (anhydrous), 0.1218: 8.6 cc. N (24.0°, 759 mm.). Subs., 0.1998: AgCl, 0.0533.
Calc. for $C_{28}H_{32}O_4N_3Cl$: N, 8.24; Cl, 6.96. Found: N, 8.11; Cl, 6.60.

Quinine o-Chloroacetylamino-benzamide, $C_{20}H_{24}O_2N_2.ClCH_2CONH\text{-}C_6H_4CONH_2(o\text{-})$.—4.25 g. of *o*-chloroacetylamino-benzamide[1] were used, with alcohol as the solvent. This was then boiled off and the gummy residue taken up in dry acetone, the chloride soon crystallizing from the dark brown, fluorescent solution. The yield was 4.7 g. The crude salt was purified by dissolving in hot, dry chloroform, treating with boneblack, filtering, and adding several volumes of dry acetone, then by recrystallization from absolute alcohol, in which it is sparingly soluble in the cold, quite easily on boiling. It forms pale tan rhombs containing 3.5 molecules of water of crystallization or 1.5 molecules of alcohol. When rapidly heated to 175°, then slowly, the anhydrous salt melts at 178–9° with gas evolution to a red-brown liquid. $[\alpha]_D^{25} = -164.4°$ in absolute alcohol, $c = 0.979$. It is soluble in dry chloroform, difficultly so in cold absolute alcohol, readily on boiling with a yellow color, and is almost insoluble in boiling benzene. A solution in dilute acid gives a gummy precipitate with excess

[1] This Journal, **39**, 1442 (1917).

sodium hydroxide and if this is washed well with water and rubbed, it gives the microcrystalline, difficultly soluble quaternary base.

Subs. (air-dry), 0.7616: loss, very slow, 0.0789 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{29}H_{33}O_4N_4Cl.3.5H_2O$: H_2O, 10.52. For $1.5C_2H_5OH$: 11.40. Found: 10.36.
Subs. (anhydrous), 0.1325: 11.9 cc. N (18.0°, 749 mm.). Subs., 0.2289: AgCl, 0.0588.
Calc. for $C_{29}H_{33}O_4N_4Cl$: N, 10.44; Cl, 6.61. Found: N, 10.39: Cl, 6.36.

(*D*) *Quaternary Salts of Quinidine.*

Quinidine Methochloride.—19.9 g. of quinidine methiodide were converted into the methochloride as usual, salting out the product cautiously with sodium chloride solution. Treated in absolute alcoholic solution after filtering from sodium chloride, with several volumes of dry ether the salt crystallizes on rubbing as aggregates of prisms containing solvent equivalent to one molecule of water of crystallization when air-dry. The yield was 8.5 g. The anhydrous salt melts with decomposition at 250–1° and gives $[\alpha]_D^{21} = +253.1°$ in water, $c = 1.505$. It dissolves readily in water, methyl or ethyl alcohol, or chloroform and is almost insoluble in dry acetone or benzene.

Subs. (air-dry), 0.5200: loss, 0.0245 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{21}H_{27}O_2N_2Cl.H_2O$: H_2O, 4.59. Found: 4.71.
Subs. (anhydrous), 0.2216: AgCl, 0.0837.
Calc. for $C_{21}H_{27}O_2N_2Cl$: Cl, 9.46. Found: 9.35.

Quinidine Benzyl Chloride.—The components were allowed to stand in a warm place in acetone solution for 15–20 days. After boiling off the acetone the residue soon began to crystallize and the process was hastened by adding dry acetone. The collected salt was ground up with dry acetone, heated to boiling, cooled, filtered again and washed with dry acetone. The yield was 85% of the quinidine used. The chloride forms pale yellow, hair-like needles, containing approximately 2 molecules of water of crystallization when air-dry. The anhydrous salt gradually melts and decomposes up to 180° when heated and gives $[\alpha]_D^{21.5} = +219.9°$ in water, $c = 0.632$. It softens under dry methyl or ethyl alcohol or dry chloroform, dissolving readily, and is more sparingly soluble in dry acetone. The chloride also dissolves readily in water.

Subs. (air-dry), 0.7596: loss, 0.0609 *in vacuo* at 80°, then 100° over H_2SO_4.
Calc. for $C_{27}H_{31}O_2N_2Cl.2H_2O$: H_2O, 7.40. Found: 8.02.
Subs. (anhydrous), 0.1350: 7.5 cc. N (24.0°, 749 mm.).
Subs., 01557: 6.70 cc. $AgNO_3$ soln. (1 cc. = 0.001812 g. Cl).
Calc. for $C_{27}H_{31}O_2N_2Cl$: N, 6.22; Cl, 7.87. Found: N, 6.29; Cl, 7.80.

Quinidine Iodoacetamide.—The quaternary salt crystallized in practically quantitative yield on suspending quinidine in hot acetone, adding one equivalent of iodoacetamide, shaking until clear, and letting stand overnight. As so obtained the salt forms a paste under water, crystallizing on rubbing. Recrystallized from water it forms rosets of long, narrow plates containing 1.5 molecules of water of crystallization. The anhydrous iodide melts to a yellow jelly at 165-70°, gradually liquefying and darkening until entirely fluid and filled with bubbles at about 190°. $[\alpha]_D^{22.5} = +166.2°$ in absolute alcohol, $c = 1.008$. It becomes pasty under methyl or ethyl alcohol, dissolving readily, and also dissolves in chloroform. The hydrate is easily soluble in hot water, sparingly in the cold, and dissolves readily in acetone, separating immediately on rubbing, presumably as a less hydrated form.

Subs. (air-dry), 0.6898: loss, 0.0330 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{22}H_{28}O_3N_3I.1.5H_2O$: H_2O, 5.04. Found: 4.78.
Subs. (anhydrous), 0.2585: AgI, 0.1180.
Calc. for $C_{22}H_{28}O_3N_3I$: I, 24.93. Found: 24.67.

Quinidine Chloroacetamide.—After recrystallization from water this salt formed rosets of prismatic needles containing one molecule of water of crystallization, the yield being about 50% of the quaternary iodide used. The anhydrous salt melts at 187–91° to a yellow jelly, gradually darkening and finally liquefying with decomposition at about 205°. $[\alpha]_D^{22} = +207.2°$ in water, $c = 1.031$. The chloride is soluble in cold water or alcohol, more readily in methyl alcohol, and very sparingly in chloroform or acetone.

Subs. (air-dry), 0.6128: loss, 0.0280 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{22}H_{28}O_3N_3Cl.H_2O$: H_2O, 4.14. Found: 4.57.
Subs. (anhydrous), 0.1300: 11.2 cc. N (21.5°, 759 mm.).
Subs., 0.2566: AgCl, 0.0861.
Calc. for $C_{22}H_{28}O_3N_3Cl$: N, 10.06; Cl, 8.49. Found: N, 9.98; Cl, 8.30.

Quinidine Iodoacetylurea.—Equimolecular amounts of quinidine and iodoacetylurea[1] were boiled in acetone until clear. The next day most of the solvent was boiled off and the residue taken up in hot water, treated with boneblack, filtered, and allowed to cool. The iodide separates in almost quantitative yield on rubbing. Recrystallized again from water it forms rosets of minute plates containing 3 molecules of water of crystallization. When rapidly heated to 165°, then slowly, the anhydrous salt melts at 170–5° to an orange mass which slowly evolves gas and blackens. It is difficultly soluble in cold water, readily in hot, and dissolves easily at room temperature in methyl or ethyl alcohol or acetone. It dissolves slowly in dry chloroform and only sparingly in benzene.

Subs. (air-dry), 0.6290: loss, 0.0532 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{23}H_{29}O_4N_4I.3H_2O$: H_2O, 8.91. Found: 8.46.
Subs. (anhydrous), 0.1360: 12.6 cc. N (30.0°, 755 mm.). Subs., 0.1658: AgI, 0.0681.
Calc. for $C_{23}H_{29}O_4N_4I$: N, 10.14; I, 22.98. Found: N, 10.37; I, 22.20.

Quinidine Chloroacetylurea.—The chloride, prepared from the iodide with silver chloride, was recrystallized from a small volume of water, forming rosets of spears containing 3.5 molecules of water of crystallization. The anhydrous salt melts and swells at 176–8°, with darkening, and gives $[\alpha]_D^{26.5} = +170.9°$ in water, $c = 1.325$. It dissolves readily in water or methyl or ethyl alcohol, less easily in dry acetone or chloroform, and difficultly in benzene. The hydrate

[1] *Iodoacetylurea*, $ICH_2CONHCONH_2$.—13.6 g. of finely powdered chloroacetylurea were added to a solution of 15 g. of dry sodium iodide in 100 cc. of dry acetone, shaken for 2.5 hours, and let stand overnight (cf. L. Finkelstein, *Ber.*, **43,** 1528 (1910)). The mixture, now a paste of minute crystals of the iodo compound, was diluted with water and filtered. The crude iodoacetylurea was recrystallized from 50% alcohol, in which it is quite sparingly soluble, even at the boiling point. 15.4 g. separated as flat prisms which melt and decompose at 182–4° with preliminary darkening and partial decomposition. The iodo compound dissolves rather difficultly in boiling water or boiling acetone and very sparingly in chloroform. It is quite soluble in cold methyl alcohol, less readily in the cold in ethyl alcohol, but more freely on boiling. It dissolves in dilute alkali and does not seem to be as active an irritant as chloroacetylurea.

Subs., 0.1510: (Kjeldahl) 18.6 cc. 0.0714 *N* HCl.
Calc. for $C_3H_5O_2N_2I$: N, 12.29. Found: 12.32.

dissolves rather sparingly in cold water, readily on heating and yields a highly supersaturated solution on cooling. Such a solution gives an oily precipitate on adding excess sodium hydroxide and evolves ammonia on boiling.

Subs. (air-dry), 0.8849: loss, 0.1041 *in vacuo* at 80° over H_2SO_4.
Calc. for $C_{23}H_{29}O_4N_4Cl.3.5H_2O$: H_2O, 12.03. Found: 11.77.
Subs. (anhydrous), 0.1418: 15.3 cc. N (28.0°, 762 mm.). Subs., 0.1457: 6.07 cc. $AgNO_3$ soln. (1 cc. = 0.001812 g. Cl).
Calc. for $C_{23}H_{29}O_4N_4Cl$: N, 12.17; Cl, 7.70. Found: N, 12.27; Cl, 7.55.

(*E*) *Quaternary Salts of Hydroquinine.*

Hydroquinine Methiodide.—Equimolecular quantities of the base[1] and methyl iodide in chloroform gave the salt in a yield of 78% of the theoretical amount, separation being completed with about two volumes of acetone. Recrystallized from absolute alcohol the methiodide forms faintly yellow prisms which are anhydrous when air-dry. It melts partially at about 170° and liquefies completely, with decomposition at 233–5°. $[\alpha]_D^{20.5}$ is − 107.6° in absolute alcohol, c = 0.855. The salt is somewhat soluble in cold water, more easily in alcohol, and readily in methyl alcohol or chloroform. Hesse[2] states that the methiodide crystallizes with one molecule of alcohol of crystallization and melts at 218° when dried.

Subs., 0.2086: AgI, 0.1048.
Calc. for $C_{21}H_{29}O_2N_2I$: I, 27.10. Found: 27.15.

Hydroquinine Methochloride.—The salt crystallizes from water as balls of hair-like needles containing 2 molecules of water of crystallization, as stated by Hesse. When rapidly heated to 155°, then slowly, the anhydrous methochloride forms a paste at 157–9°, and on further heating finally becomes a yellow liquid filled with bubbles at 172–3°. Hesse gives the melting point as 168°. $[\alpha]_D^{21.5}$ in water is − 162.9°, c = 1.470. The anhydrous salt also dissolves very easily in methyl or ethyl alcohol, less readily in dry acetone.

[1] Ger. pat. 252,136; This Journal, **41**, 819 (1919).
[2] *Ann.*, **241**, 275 (1887).

Subs. (anhydrous), 0.2760: AgCl, 0.1052.
Calc. for $C_{21}H_{29}O_2N_2Cl$: Cl, 9.41. Found: 9.43.

Hydroquinine Benzyl Chloride.—The components were boiled for about 15 hours in dry acetone. After long standing the acetone was boiled off and the dark residue taken up in dry methyl alcohol. The chloride crystallized rapidly on adding several volumes of dry ether and was recrystallized from water, separating slowly on seeding at room temperature as a crust of rhombs and prisms containing one molecule of water of crystallization. An additional quantity of equal purity was obtained by adding saturated sodium chloride solution to the aqueous mother liquor, letting stand, and washing the crystals with cold water. The total yield was 3 g., starting with 3.6 g. of the base. When rapidly heated to 200°, then slowly, the anhydrous chloride melts at 202–3.5° to a dark red, viscous liquid which soon decomposes. $[\alpha]_D^{23}$ in water is − 196.9°, c = 0.787. It dissolves readily in methyl or ethyl alcohol or chloroform, less easily in dry acetone, and melts under hot benzene, dissolving with difficulty. The air-dry salt dissolves rather sparingly in cold water, quite readily on boiling, the solution having a weak, bitter taste. A not too dilute aqueous solution gives immediate precipitates of the nitrate and permanganate with the appropriate salts. Solutions in dil. sulfuric or nitric acid have a marked blue fluorescence, while the solution in dil. hydrochloric acid is pale yellow and does not fluoresce.

Subs. (air-dry), 0.7928: loss, 0.0281 *in vacuo* at 100° over H_2SO_4. Subs., 0.1238: 7.10 cc. N (25.5°, 755 mm.). Subs., 0.1520: (Kjeldahl, with H_2SO_4, K_2SO_4, and crystal of $CuSO_4$) 8.8 cc. 0.0714 *N* HCl. Subs., 0.1203: 5.1 cc. $AgNO_3$ soln. (1 cc. = 0.001794 g. Cl).
Calc. for $C_{27}H_{33}O_2N_2Cl.H_2O$: H_2O, 3.83; N, 5.95; Cl, 7.53. Found: H_2O, 3.54; N, 6.51, 5.79; Cl, 7.61.

Hydroquinine Iodoacetamide.—Acetone was used as solvent and after several hours it was boiled off and hot water added, the iodide crystallizing on cooling and rubbing as prismatic plates. The yield was almost quantitative. Recrystallized from water the salt forms prismatic needles containing between 2.5 and 3 molecules of water of crystallization. The anhydrous salt gradually softens to a yellow jelly above 145°, deepening in color as the temperature is raised, and

becoming entirely fluid at about 185°. $[\alpha]_D^{22.5}$ in absolute alcohol is $-121.5°$, $c = 0.976$. The dried salt also softens and dissolves readily in dry methyl or ethyl alcohol, dry acetone, or dry chloroform.

Subs. (air-dry), 0.6120: loss, 0.0534 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{22}H_{30}O_3N_3I.2.5H_2O$: H_2O, 8.10. Found: 8.72.
Subs. (anhydrous), 0.2039: AgI, 0.0933.
Calc. for $C_{22}H_{30}O_3N_3I$: I, 24.83. Found: 24.72.

Hydroquinine Chloroacetamide.—Instead of adding sodium chloride to the filtrate from the silver iodide precipitate the solution was allowed to stand overnight, filtered from a small amount of a substance crystallizing in silky needles, and the filtrate concentrated to dryness *in vacuo*. The residue was taken up in absolute alcohol, concentrated to dryness *in vacuo*, again taken up in absolute alcohol, filtered from a slight turbidity with the aid of boneblack, and concentrated as before, giving a friable, white, amorphous residue which could easily be removed from the flask. The dried salt turns yellow and softens at about 160–75° to a jelly which gradually darkens and liquefies completely with gas evolution at about 195°. $[\alpha]_D^{23} = -129.1°$ in water, $c = 0.852$. The product is very easily soluble in acetone or chloroform and also dissolves readily in cold water.

Subs. (anhydrous), 0.1356: 12.2 cc. N (27.5°, 758 mm.).
Subs., 0.1451: 6.92 cc. $AgNO_3$ soln. (1 cc. = 0.001812 g. Cl).
Calc. for $C_{22}H_{30}O_3N_3Cl$: N, 10.02; Cl, 8.45. Found: N, 10.19; Cl, 8.64.

Hydroquinine Chloroacet-methylamide, $C_{20}H_{26}O_2N_2.ClCH_2CONHCH_3$.—This salt was obtained directly from chloroacet-methylamide[1] in boiling dry acetone. As the produce could neither be crystallized nor converted into a crystalline bromide or hydrochloride it was precipitated with dry ether, dried *in vacuo* at room temperature over sulfuric acid, and pulverized. The salt intumesces at 110–20°, gradually melting above this to a glassy mass by the time 160° is reached, and forming a dark brown liquid at about 200°. It dissolves readily in methyl or ethyl alcohol, acetone, chloroform, benzene, or water.

Subs., 0.1340: 10.4 cc. N (18.0°, 764 mm.).
Subs., 0.1165: 5.09 cc. $AgNO_3$ soln. (1 cc. = 0.001794 g. Cl).
Calc. for $C_{23}H_{32}O_3N_3Cl$: N, 9.69; Cl, 8.17. Found: N, 9.16; Cl, 7.84.

[1] THIS JOURNAL, **41**, 472 (1919).

Hydroquinine Chloroacet-ethylamide.—This salt was prepared in the same way, using chloroacet-ethylamide.[1] On adding dry ether the gummy material crystallized partially, but as the crystals softened to a gum on desiccation or air-drying, and as an attempt at recrystallization by dissolving in dry acetone and precipitating with dry ether proved no better, the substance was left in the crude state and dried *in vacuo* over sulfuric acid. It melted to a yellowish jelly at 110–35°, becoming completely fluid at about 160°. In solubility it resembles the methyl compound.

Subs., 0.1680: 13.7 cc. N (23.0°, 751 mm.). Subs., 0.1561: AgCl, 0.0479.
Calc. for $C_{24}H_{34}O_3N_3Cl$: N, 9.39; Cl, 7.92. Found: N, 9.30; Cl, 7.59.

Hydroquinine Chloroacet-dimethylamide, $C_{20}H_{26}O_2N_2.ClCH_2CON(CH_3)_2$.—An amorphous salt was obtained from chloroacet-dimethylamide,[2] melting partially and intumescing at 130–40°, becoming transparent at about 180°, and liquefying completely at about 200°. It resembles the monomethyl and -ethyl derivatives in its solubility relationships.

Subs., 0.1362: 10.4 cc. N (19.5°, 765 mm.).
Subs., 0.1797: 7.68 cc. $AgNO_3$ soln. (1 cc. = 0.001794 g. Cl).
Calc. for $C_{24}H_{34}O_3N_3Cl$: N, 9.39; Cl, 7.92. Found: N, 8.97; Cl, 7.67.

Hydroquinine Chloroacet-diethylamide, $C_{20}H_{26}O_2N_2.ClCH_2CON(C_2H_5)_2$.—1.6 g. of chloroacet-diethylamide[3] were used. On adding ligroin until the initial turbidity just redissolved the chloride crystallized on standing as rosets of prismatic needles, precipitation being completed by the addition of more ligroin and letting stand. The yield was 3.5 g. On dissolving in not too little dry chloroform and treating cautiously with ligroin, seeding, and adding more ligroin from time to time the salt forms minute prisms containing solvent approximately equivalent to one molecule of water of crystallization. The anhydrous salt darkens somewhat above 205° and melts and decomposes at 209–10°. $[\alpha]_D^{27.5} = -84.8°$ in water, $c = 0.854$. It dissolves readily at room temperature in water, methyl or ethyl alcohol,

[1] *J. Biol. Chem.*, **21**, 149 (1915).
[2] *Ibid.*, **21**, 148 (1915).
[3] *Ibid.*, **21**, 149 (1915).

or chloroform, sparingly in cold acetone, more easily on heating, and is almost insoluble in benzene. It has a sharp, bitter taste.

Subs. (air-dry), 0.6028: loss, 0.0182 *in vacuo* at 80° over H_2SO_4.
Calc. for $C_{26}H_{38}O_3N_3Cl.H_2O$: H_2O, 3.65. Found: 3.02.
Subs. (anhydrous), 0.1226; 10.0 cc. N (30.0°, 757 mm.). Subs., 0.1247: 5.15 cc. $AgNO_3$ soln. (1 cc. = 0.00181 g. Cl).
Calc. for $C_{26}H_{38}O_3N_3Cl$: N, 8.84; Cl, 7.45. Found: N, 9.16; Cl, 7.48.

Hydroquinine Chloroacetyl-benzylamine, $C_{20}H_{26}O_2N_2.ClCH_2CONHCH_2C_6H_5$.—Starting with chloroacetyl-benzylamine[1] in dry acetone the salt crystallized from the reaction mixture. Recrystallized from butyl alcohol[2] the chloride separates as delicate, glistening needles. The anhydrous salt melts at 197–8° to an orange liquid containing bubbles. $[\alpha]_D^{23}$ in water is $-42.5°$, $c = 0.670$. The compound dissolves fairly readily in water, easily in methyl or ethyl alcohol, or chloroform. A dilute aqueous solution gives a precipitate with sodium hydroxide.

Subs., 0.1409: 10.2 cc. N (23.0°, 744 mm.). Subs., 0.1697: 6.45 cc. $AgNO_3$ soln. (1 cc. = 0.00181 g. Cl).
Calc. for $C_{29}H_{34}O_3N_3Cl$: N, 8.29; Cl, 6.99. Found: N, 8.18; Cl, 6.88.

Hydroquinine Chloroacetanilide.—On adding hot water to the residue after boiling off the acetone the crude product separated at first oily on cooling but soon crystallized in almost quantitative yield. Recrystallized from 50% alcohol the chloride forms rosets of delicate needles containing 3 molecules of water of crystallization. When anhydrous it softens to an orange jelly at 160–70°, liquefying completely at about 210° with darkening and slow gas evolution. $[\alpha]_D^{24} = -95.9°$ in absolute alcohol, $c = 0.824$. It dissolves readily in methyl or ethyl alcohol, acetone, chloroform, or benzene, and only sparingly in dry ether. The hydrate is difficultly soluble in cold water, more readily on heating, and is rather sparingly soluble in cold 50% alcohol. It dissolves in 10% aqueous hydrochloric acid, the *hydrochloride* soon separating as sheaves of minute needles.

Subs. (air-dry), 0.5450: loss, 0.0534 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{28}H_{34}O_3N_3Cl.3H_2O$: H_2O, 9.83. Found: 9.80.

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[1] *J. Biol. Chem.*, **20**, 686 (1915).
[2] We have found commercial butyl alcohol to be a very useful solvent.

Subs. (anhydrous), 0.1469: 11.0 cc. N (26.0°, 762 mm.). Subs., 0.1556: AgCl, 0.0443.
Calc. for $C_{28}H_{34}O_3N_3Cl$: N, 8.48; Cl, 7.15. Found: N, 8.58; Cl, 7.05.

Hydroquinine Acetanilide Dinitrate, $C_{20}H_{26}O_2N_2.O_3NCH_2CONHC_6H_5.HNO_3$.—One g. of hydroquinine chloroacetanilide was dissolved in about 100 cc. of boiling water, and a few drops of dil. nitric acid added on cooling. The solution was chilled, acidified strongly with dil. nitric acid and rubbed, 0.6 g. of the dinitrate separating as long, delicate needles. The salt was washed with a little ice-cold water and air-dried, containing then approximately 2 molecules of water of crystallization. When heated the anhydrous salt melts to a jelly at about 145–50° with slight preliminary softening, then darkens and intumesces. It softens under the usual anhydrous solvents, dissolving readily in dry methyl or ethyl alcohol or dry acetone, sparingly in dry chloroform, even on boiling, and is practically insoluble in boiling benzene. The hydrate dissolves very sparingly in cold water, fairly readily on boiling, with a faint greenish yellow color.

Subs. (air-dry), 0.5877: loss, 0.0317 *in vacuo* at room temp. over H_2SO_4 + NaOH.
Calc. for $C_{28}H_{34}O_3N_3.NO_3.HNO_3.2H_2O$: H_2O, 5.80. Found: 5.39.
Subs. (anhydrous), 0.1265: 13.4 cc. N (23.0°, 755 mm.).
Calc. for $C_{28}H_{34}O_3N_3.NO_3.HNO_3$: N, 11.97. Found: 12.14.

Hydroquinine m-Nitro-chloroacetanilide.—The gelatinous reaction mixture was disintegrated with hot water and let stand. The resulting precipitate, recrystallized first from 25% alcohol, then from 50% alcohol, separated as pale drab-colored, minute needles which contained approximately 2 molecules of water of crystallization. When slowly heated, the anhydrous salt begins to soften at 150°, gradually melting to a brownish jelly up to 160°, and liquefying completely and decomposing at 195–200°. It softens under and dissolves readily with a pale yellow color in dry methyl or ethyl alcohol, dry acetone, dry chloroform, or ethyl acetate, dissolving sparingly in cold benzene and readily on boiling. The hydrate is quite difficultly soluble in cold water, more easily on boiling. An aqueous suspension turns bright yellow and flocculates with a few drops of aqueous sodium hydroxide.

Subs. (air-dry), 0.5443: loss, 0.0374 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{28}H_{33}O_5N_4Cl.2H_2O$: H_2O, 6.25. Found: 6.87.
Subs. (anhydrous), 0.1279: 12.0 cc. N (24.0°, 759 mm.). Subs., 0.1175: 4.05 cc. $AgNO_3$ soln. (1 cc. = 0.001794 g. Cl).
Calc. for $C_{28}H_{33}O_5N_4Cl$: N, 10.36; Cl, 6.56. Found: N, 10.77; Cl, 6.19.

Hydroquinine m-Chloroacetylamino-acetanilide, $C_{20}H_{26}O_2N_2.ClCH_2CONHC_6H_4NHCOCH_3(m\text{-})$.—Starting with *m*-chloroacetylamino-acetanilide,[1] a gummy quaternary salt separated, from which the acetone was eventually poured off and the residue boiled with dry methyl ethyl ketone. The gum which separated on cooling was filtered off and on letting stand and rubbing the chloride separated from the filtrate as practically colorless, radiating masses of minute crystals. A further quantity was obtained by again extracting the crude gummy material with the filtrate. The air-dry salt contained solvent of crystallization corresponding to 3 to 3.5 molecules of water. When heated the anhydrous salt gradually melts to an orange mass above 150°, with slow gas evolution, becoming completely fluid at about 205–10°. It first softens under, and then dissolves freely in dry methyl or ethyl alcohol, also dissolves readily in chloroform, and is very difficultly soluble in boiling dry acetone, benzene, or ethyl acetate.

Subs. (air-dry), 0.4457: loss, 0.0432 *in vacuo* at 80° over H_2SO_4.
Calc. for $C_{30}H_{37}O_4N_4Cl.3H_2O$: H_2O, 8.91. Found: 9.69.
Subs. (anhydrous), 0.1309: 11.25 cc. N (21.5°, 755 mm.). Subs., 0.1412: 4.92 cc. $AgNO_3$ soln. (1 cc. = 0.001794 g. Cl).
Calc. for $C_{30}H_{37}O_4N_4Cl$: N, 10.13; Cl, 6.41. Found: N, 9.90; Cl, 6.25.

Hydroquinine p-Chloroacetylamino-acetanilide.—In the case of the sparingly soluble *p*-chloroacetylamino-acetanilide[2] absolute alcohol was used as solvent. After letting stand in the cold the insoluble material was filtered off, and the filtrate concentrated to small bulk. The residue was taken up in boiling water, treated with boneblack and the filtrate cooled, diluting so that it became only faintly turbid at room temperature. After filtering again the solution was seeded with crystals obtained by adding sodium chloride to a test portion. The crude quaternary salt was recrystallized from water, separating as rosets and sheaves of delicate, silky needles. Additional amounts were obtained

[1] This Journal, **39**, 1448 (1917).
[2] *Ibid.*, **39**, 1455 (1917).

by adding sodium chloride to the mother liquors. The air-dry salt contains 3 molecules of water of crystallization and dissolves sparingly in cold water, more easily on heating. The anhydrous chloride begins to soften at about 160° and darkens and becomes completely fluid, with slow gas evolution, at about 205°. It dissolves readily in methyl or ethyl alcohol, acetone, or chloroform, and only sparingly in boiling benzene or ethyl acetate. Analysis indicated that the substance was not entirely pure.

Subs. (air-dry), 0.5336: loss, 0.0477 *in vacuo* at room temp. over H_2SO_4.
Calc. for $C_{30}H_{37}O_4N_4Cl.3H_2O$: H_2O, 8.91. Found: 8.94.
Subs. (anhydrous), 0.1403: 12.0 cc. N (22.0°, 751 mm.). Subs., 0.1260: 4.0 cc. $AgNO_3$ soln. (1 cc. = 0.001794 g. Cl).
Calc. for $C_{30}H_{37}O_4N_4Cl$: N, 10.13; Cl, 6.41. Found: N, 9.79; Cl, 5.70.

Hydroquinine p-Chloroacetylamino-dimethylaniline, $C_{20}H_{26}O_2N_2.ClCH_2CONHC_6H_4N(CH_3)_2(p\text{-})$.—After boiling off the acetone the residue, using 3.5 g. of base, was taken up in about 500 cc. of boiling water and treated with boneblack, yielding 4.3 g. of the crystalline salt on cooling. Recrystallized from 50% alcohol it separates as aggregates of minute, cream-colored needles containing 2.5 molecules of water of recrystallization and dissolving very difficultly in cold water, quite easily on boiling. An aqueous suspension gives a brownish rose color with ferric chloride. The anhydrous salt gradually melts to a jelly at about 165°, becoming completely fluid, with partial decomposition at about 200°. $[\alpha]_D^{13.5} = -82.9°$ in absolute alcohol, $c = 1.037$. It softens under and dissolves freely in dry methyl or ethyl alcohol, dry acetone, or dry chloroform.

Subs. (air-dry), 0.5172: loss, 0.0407 *in vacuo* first at room temp., then 80° over H_2SO_4.
Calc. for $C_{30}H_{39}O_3N_4Cl.2.5H_2O$: H_2O, 7.72. Found: 7.87.
Subs. (anhydrous), 0.1185: 10.4 cc. N (20.5°, 763 mm.). Subs., 0.1196: 4.2 cc. $AgNO_3$ soln. (1 cc. = 0.001794 g. Cl).
Calc. for $C_{30}H_{39}O_3N_4Cl$: N, 10.39; Cl, 6.58. Found: N, 10.25; Cl, 6.30.

Hydroquinine p-Chloroacetylamino-diethylaniline.—In the case of *p*-chloroacetylamino-diethylaniline[1] the reaction mixture was diluted with dry acetone and the salt precipitated as amorphous flocks with

[1] *J. Biol. Chem.*, **21**, 115 (1915).

the aid of dry ether. The product was filtered off, washed with a little dry ether, dried, and then dissolved in hot ethyl acetate and seeded while still warm with a few of the crystals left in the reaction flask on evaporation of the solvent. On letting stand in a warm place the salt separated slowly as sheaves of delicate needles. The air-dry product still possessed the odor of ethyl acetate. The anhydrous salt begins to soften at about 150°, and when slowly heated above this point forms a transparent jelly at 165–70°, and a red fluid at 190–5°, with slight gas evolution. $[\alpha]_D^{20} = -80.5°$ in absolute alcohol, $c = 1.118$. It dissolves readily in methyl or ethyl alcohol, acetone, chloroform, or benzene, and also in boiling ethyl acetate, although sparingly in the cold. It is very difficultly soluble in cold water, more easily on boiling. An aqueous suspension gives a slowly-developing salmon color with ferric chloride.

Subs. (anhydrous), 0.1277: 11.0 cc. N (20.5°, 757 mm.). Subs., 0.1034: 3.64 cc. $AgNO_3$ soln. (1 cc. = 0.001794 g. Cl).
Calc. for $C_{32}H_{43}O_3N_4Cl$: N, 9.89; Cl, 6.26. Found: N, 9.99; Cl, 6.32.

Hydroquinine o-Chloroacetylamino-phenol.—To the reaction mixture from *o*-chloroacetylamino-phenol,[1] a few cc. of absolute alcohol were added and then dry ether and ligroin to incipient turbidity. The quaternary salt crystallized on rubbing and letting stand. Recrystallized by dissolving in hot absolute alcohol and adding dry ether until just turbid, it separated on seeding as rosets of long, flat platelets containing solvent equivalent to 1.5 molecules of water of crystallization. It dissolves sparingly in cold water, more freely on heating, separating as a jelly on cooling. The anhydrous salt gradually melts to a jelly above 155°, finally reddens, and becomes completely fluid and evolves gas at 185°. It dissolves very easily in dry methyl alcohol and less readily in absolute alcohol or dry acetone. An alkaline suspension couples with diazotized sulfanilic acid.

Subs. (air-dry), 0.5750: loss, 0.0308 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{28}H_{34}O_4N_3Cl.1.5H_2O$: H_2O, 5.01. Found: 5.36.
Subs. (anhydrous), 0.1449: 10.35 cc. N (20.5°, 758 mm.). Subs., 0.1539: AgCl, 0.0469.
Calc. for $C_{28}H_{34}O_4N_3Cl$: N, 8.21; Cl, 6.93. Found: N, 8.29; Cl, 7.54.

[1] This Journal, **41**, 458 (1919).

Hydroquinine m-Chloroacetylamino-phenol.—Starting with 1.9 g. of *m*-chloroacetylamino-phenol[1] 4.5 g. of the salt crystallized from the reaction mixture as hard nodules. Recrystallized from not too little 50% alcohol, seeding and rubbing while still warm, the chloride separates as cream-colored aggregates of microscopic leaflets containing 3 molecules of water of crystallization. If care is not taken the solution gelatinizes. The anhydrous salt softens and sinters above 160°, melts to a reddish jelly at about 180–5°, and is completely fluid, with slow gas evolution, at about 205°. $[\alpha]_D^{21} = -115.7°$ in absolute alcohol, $c = 0.800$. It first softens under, then resolidifies, dissolving freely, in absolute alcohol or dry methyl alcohol, also softening under boiling dry acetone and dissolving with difficulty. The salt is very difficultly soluble in cold water, more easily on boiling, and is readily soluble in hot 50% alcohol, quite sparingly in the cold.

Subs. (air-dry), 0.5070: loss, 0.0489 *in vacuo* at 80° over H_2SO_4.
Calc. for $C_{28}H_{34}O_4N_3Cl.3H_2O$: H_2O, 9.56. Found: 9.64.
Subs. (anhydrous), 0.1262: 0.55 cc. N (26.0°, 754 mm.). Subs., 0.1535: AgCl, 0.0409.
Calc. for $C_{28}H_{34}O_4N_3Cl$: N, 8.21; Cl, 6.93. Found: N, 8.57; Cl, 6.59.

The Hydrochloride.—The purified salt was rubbed in a mortar with 1:1 hydrochloric acid, yielding a pasty mass which eventually crystallized. Recrystallized from hot water containing a little hydrochloric acid it separates on cooling and seeding as cream-colored prismatic needles and short rods, separation being completed by further small additions of 10% hydrochloric acid. The hydrochloride contains 4.5 molecules of water of crystallization and dissolves fairly readily in water. The anhydrous salt is a pale sulfur-yellow and forms an orange jelly at 195–7°, melting and evolving gas at about 200°. It dissolves readily in methyl or ethyl alcohol and is practically insoluble in dry acetone. It does not dissolve in excess dil. alkali, the suspension coupling with diazotized sulfanilic acid.

Subs. (air-dry), 0.5030: loss, 0.0650.
Calc. for $C_{28}H_{34}O_4N_3Cl.HCl.4.5H_2O$: H_2O, 12.88. Found: 12.92.
Subs. (anhydrous), 0.1654: AgCl, 0.0860.
Calc. for $C_{28}H_{34}O_4N_3Cl.HCl$: Cl, 12.93. Found: 12.86.

[1] This Journal, **39**, 1442 (1917).

Hydroquinine p-Chloroacetylamino-phenol Hydrochloride.—In the case of *p*-chloroacetylamino-phenol[1] a portion of the quaternary addition product separated as a gum, followed on standing by radiating masses of fibrous needles and the ultimate crystallization of the gum. As the salt could not be recrystallized it was converted into the hydrochloride by dissolving in a slight excess of very dilute hydrochloric acid, adding the conc. acid, with cooling, until slightly turbid, treating with boneblack, and again adding conc. hydrochloric acid to the filtrate until the initial turbidity just redissolved. The hydrochloride separated on seeding with crystals obtained from a test portion, precipitation being completed by the further careful addition of conc. hydrochloric acid. Recrystallized from warm water, adding conc. hydrochloric acid to the cooled solution until just turbid, the hydrochloride crystallized as cream-colored rosets of prismatic needles containing 4.5 molecules of water of crystallization, completing the separation as before. The yield was 7.2 g. The salt dissolves fairly readily in water at room temperature and is readily salted out by the addition of hydrochloric acid. An aqueous solution gives a dull, grayish color with ferric chloride. The anhydrous salt is darker than the hydrate and when rapidly heated to 195° then slowly, melts at 196–7° to a red-brown jelly which soon evolves gas. It turns gummy under dry methyl or ethyl alcohol, dissolving readily, and also softens under dry chloroform but remains insoluble. It is also practically insoluble in dry acetone.

Subs. (air-dry), 0.4937: loss, 0.0632.
Calc. for $C_{28}H_{34}O_4N_3Cl.HCl.4.5H_2O$: H_2O, 12.88. Found: 12.80.
Subs. (anhydrous), 0.1414: 9.4 cc. N (24.5°, 759 mm.). Subs., 0.1744: AgCl, 0.0907.
Calc. for $C_{28}H_{34}O_4N_3Cl.HCl$: N, 7.67; Cl, 12.93. Found: N, 7.62; Cl, 12.86.

Hydroquinine 4-Chloroacetylamino-pyrocatechol Hydrochloride, $C_{20}H_{26}O_2N_2.ClCH_2CONHC_6H_3(OH)_2.HCl(3,4-)$.—After pouring off the acetone from the gummy precipitate in the case of 4-chloroacetylamino-pyrocatechol[2] 10% aqueous hydrochloric acid was added, as well as a little alcohol to facilitate solution. On cautiously adding further portions of hydrochloric acid and treating with boneblack to remove the

[1] *Loc. cit.*
[2] This Journal, **41**, 468 (1919).

first gummy precipitates a clear, red-brown solution was finally obtained. This was seeded with crystals obtained by rubbing a portion of the crude gummy salt with 1:1 hydrochloric acid and letting stand. The hydrochloride separated in poor yield as pale yellow needles containing 4.5 molecules of water of crystallization, precipitation being completed by finally adding more acid. The salt dissolves in water, the solution giving an olive-green color with ferric chloride, changing rapidly to yellow-brown. An aqueous solution gave a precipitate with sodium hydroxide, insoluble in excess. When rapidly heated to 195°, then slowly, the anhydrous hydrochloride melts to a jelly at 196–8°, gradually decomposing as the temperature is further raised. It dissolves readily in methyl alcohol, more slowly in absolute alcohol, and is practically insoluble in dry acetone or dry chloroform.

Subs. (air-dry), 0.4249: loss, 0.0530.
Calc. for $C_{28}H_{34}O_5N_3Cl.HCl.4.5H_2O$: H_2O, 12.57. Found: 12.48.
Subs. (anhydrous), 0.1176: 7.4 cc. N (21.5°, 767 mm.). Subs., 0.1758: AgCl, 0.0877.
Calc. for $C_{28}H_{34}O_5N_3Cl.HCl$: N, 7.45; Cl, 12.57. Found: N, 7.36; Cl, 12.34.

Hydroquinine Chloroacetyl-o-anisidine.—Starting with 4 g. of chloroacetyl-*o*-anisidine,[1] 7 g. of the crude quaternary salt were finally deposited from the ice-cold solution on seeding with crystals obtained by spontaneous evaporation. The substance was recrystallized from butyl alcohol, letting stand in the ice box, and seeding, 6.4 g. separating as radiating, hair-like needles. The anhydrous compound gradually sinters and softens to a jelly above 110°, turns yellow above 150°, and is completely fluid at about 185°. $[\alpha]_{32} = -72.5°$ in absolute alcohol, $c = 0.904$. It softens under and dissolves readily in dry methyl or ethyl alcohol, dry chloroform, or dry acetone, dissolves less readily in benzene, and gives a pale yellow color in conc. sulfuric acid. The air-dry salt dissolves slowly but freely in water.

Subs. (anhydrous), 0.1268: 9.2 cc. N (29.5°, 755 mm.). Subs., 0.1337: 5.07 cc. $AgNO_3$ soln. (1 cc. = 0.00181 g. Cl).
Calc. for $C_{29}H_{36}O_4N_3Cl$: N, 7.99; Cl, 6.75. Found: N, 8.14; Cl, 6.87.

Hydroquinine Chloroacetyl-m-anisidine.—(From 2 g. of chloroacetyl-*m*-anisidine.[2]) The solvent was boiled off and the residue taken up in

[1] This Journal, **41**, 1451 (1919).
[2] *Ibid.*, **41**, 1452 (1919).

50% alcohol and cautiously diluted with hot water, filtering off the slight turbidity which first formed. On seeding the solution with crystals obtained by several days' standing of a dilute solution of the crude material in hot water, the salt gradually crystallized, especially on the further addition of small quantities of water and occasional warming. Recrystallized by dissolving in alcohol, diluting with ether, and seeding, the quaternary salt separates as radiating aggregates of delicate needles containing solvent equivalent to 3 molecules of water of crystallization when air-dry. The anhydrous salt gradually sinters and softens above 140° and forms an orange fluid at about 190°. $[\alpha]_D^{23} = -102.9°$ in absolute alcohol, $c = 1.292$. It melts under and dissolves readily in the following dry solvents: methyl or ethyl alcohol, chloroform, acetone, or benzene; and dissolves partly in cold water, the solution foaming readily and soon setting to a jelly. It is readily soluble in boiling water.

Subs. (air-dry), 0.7289: loss, 0.0715 *in vacuo* at 80° over H_2SO_4.
Calc. for $C_{29}H_{36}O_4N_3Cl.3H_2O$: H_2O, 9.32. Found: 9.81.
Subs. (anhydrous), 0.1489: 10.5 cc. N (25.5°, 760 mm.). Subs., 0.1085: 3.9 cc. $AgNO_3$ soln. (1 cc. = 0.001794 g. Cl).
Calc. for $C_{29}H_{36}O_4N_3Cl$: N, 7.99; Cl, 6.75. Found: N, 8.07; Cl, 6.45.

The Hydrochloride.—3.5 g. of the quaternary salt were dissolved in absolute alcohol containing an excess of dry hydrochloric acid and treated with dry ether until a faint turbidity persisted. On rubbing and letting stand 3.2 g. of the hydrochloride separated slowly as pale yellow, radiating aggregates of rhombic crystals. When rapidly heated to 165°, then slowly, the salt begins to soften, and melts at 170–1° with gas evolution. It dissolves readily in methyl alcohol or chloroform, less easily in absolute alcohol, and only sparingly in hot, dry acetone, the insoluble portion changing to a gummy mass. As obtained above the hydrochloride apparently retains 2 molecules of water of crystallization. An attempt to drive this off at 80° *in vacuo*, resulted in the loss of hydrochloric acid.

Subs., 0.1487: 9.0 cc. N (22.5°, 765 mm.). Subs., 0.1201: 7.92 cc. $AgNO_3$ soln. (1 cc. = 0.001794 g. Cl).
Calc. for $C_{29}H_{36}O_4N_3Cl.HCl.2H_2O$: N, 7.03; Cl, 11.85. Found: N, 7.05; Cl, 11.83.

Hydroquinine Chloroacetyl-p-anisidine.—After boiling off the acetone the residue was taken up in hot commercial butyl alcohol. The filtrate deposited radiating masses of delicate needles and the separation was completed by the cautious addition of dry ether. The salt was recrystallized by dissolving in dry acetone, adding ligroin until the initial turbidity just dissolved, and seeding. When air-dry it retained an amount of solvent equivalent to between 1 and 1.5 molecules of water of crystallization. The anhydrous salt gradually softens to a jelly above 150° and is completely fluid at about 190°. $[\alpha]_D^{20} = -93.9°$ in absolute alcohol, $c = 1.128$. The salt dissolves very readily in methyl or ethyl alcohol or chloroform, somewhat less easily in dry acetone, and very sparingly in dry ether. It is difficultly soluble in cold water or benzene, readily on boiling, and separates from the former as a jelly on cooling. It dissolves in conc. sulfuric acid with a dull yellow color.

Subs. (anhydrous), 0.1424: 10.0 cc. N (18.5°, 745 mm.). Subs., 0.1461: 5.20 cc. $AgNO_3$ soln. (1 cc. = 0.00181 g. Cl).
Calc. for $C_{29}H_{36}O_4N_3Cl$: N, 7.99; Cl, 6.75. Found: N, 8.06; Cl, 6.45.

The Hydrochloride.—The quaternary salt was dissolved in a small volume of absolute alcohol, treated with absolute alcoholic hydrochloric acid, and seeded with crystals obtained by manipulating a test portion with dry ether. The hydrochloride separated rapidly on rubbing as thick, yellow, hexagonal, microscopic plates. When rapidly heated to 180°, then slowly, the substance melts at 180–4° to an orange mass containing bubbles and becomes completely fluid and decomposes at about 190°. It is rather sparingly soluble in the cold in absolute alcohol, easily on warming, and dissolves readily in dry methyl alcohol. The aqueous solution foams readily.

Subs., 0.1051: 7.40 cc. $AgNO_3$ soln. (1 cc. = 0.001794 g. Cl).
Calc. for $C_{29}H_{36}O_4N_3Cl.HCl$: Cl, 12.61. Found: 12.64.

Hydroquinine Chloroacetyl-o-phenetidine, $C_{20}H_{26}O_2N_2.ClCH_2CONH\text{-}C_6H_4OC_2H_5(o\text{-})$.—In the case of chloroacetyl-*o*-phenetidine[1] the acetone was boiled off and the residue taken up in boiling water and treated with boneblack. As the salt separated from the filtrate as an oil on rapid chilling it was necessary to cool slowly and seed with

[1] This Journal, **41**, 1452 (1919).

crystals obtained by adding sodium chloride to a diluted test portion. Recrystallized from water it separates very slowly on seeding as rosets and sheaves of delicate needles containing 2.5 molecules of water of crystallization. Additional quantities were recovered by treating the mother liquors carefully with saturated salt solution. The anhydrous salt softens above 125°, forms a transparent jelly at about 140° and a yellow fluid at 170–2°. It dissolves readily in methyl or ethyl alcohol, chloroform, or acetone, and only very sparingly in dry ether.

Subs. (air-dry), 0.7096: loss, 0.0573 *in vacuo* at room temp. over H_2SO_4.
Calc. for $C_{30}H_{38}O_4N_3Cl.2.5H_2O$: H_2O, 7.70. Found: 8.08.
Subs. (anhydrous), 0.1531: 11.0 cc. N (23.5°, 744 mm.). Subs., 0.1317: 4.45 cc. $AgNO_3$ soln. (1 cc. = 0.001794 g. Cl).
Calc. for $C_{30}H_{38}O_4N_3Cl$: N, 7.79; Cl, 6.57. Found: N, 8.10; Cl, 6.07.

Hydroquinine Chloroacetyl-m-phenetidine Hydrochloride.—(From 2.2 g. of chloroacetyl-*m*-phenetidine.[1]) As the quaternary salt itself could not be obtained crystalline the acetone was boiled off and the residue taken up in absolute alcohol and treated with an excess of absolute alcoholic hydrochloric acid. The salt crystallized after adding dry ether to incipient turbidity and rubbing. The supernatant solution was then decanted off and the precipitate washed twice by decantation with dry alcohol-ether mixture containing a little hydrochloric acid. After dissolving the residue in boiling absolute alcohol, filtering hot from traces of impurities, and adding a little absolute alcoholic hydrochloric acid, the salt separated when seeded as lemon-yellow, rhombic crystals, which are hygroscopic when moist. 4.2 g. were obtained, melting at 173–4° with preliminary sintering and softening. As so obtained the hydrochloride apparently retained 2 molecules of water of crystallization or one molecule of alcohol. It dissolves readily in water, a concentrated solution soon setting to a jelly. It also dissolves readily in chloroform and lightens in color and turns pasty under boiling dry acetone, possibly owing to dehydration. It dissolves sparingly in cold absolute alcohol, more easily on warming.

Subs., 0.1428; 8.3 cc. N (22.0°, 773 mm.). Subs., 0.1261: 8.15 cc. $AgNO_3$ soln. (1 cc. = 0.001794 g. Cl).
Calc. for $C_{30}H_{38}O_4N_3Cl.HCl.2H_2O$: N, 6.87; Cl, 11.58. Found: N, 6.85; Cl, 11.60.

[1] This Journal, **41**, 1452 (1919).

Hydroquinine Chloroacetyl-p-phenetidine.—After removing the acetone the residue was taken up in boiling water, treated with boneblack, and the filtrate seeded with crystals obtained by slow, spontaneous evaporation of a test portion of the original solution. The salt was recrystallized from water, forming sheaves of delicate needles containing 2.5 molecules of water of crystallization, dissolving with difficulty in water, and giving $[\alpha]_D^{27.5} = -89.1°$ in 95% alcohol, $c = 1.033$. The anhydrous salt softens slightly above 110°, sinters to a jelly at 150–60°, turns yellow, slowly evolves gas, and becomes more fluid as the temperature is further raised, and is completely molten at 210°. It is very easily soluble in absolute alcohol, acetone, chloroform, or benzene, and only difficultly in dry ether. It gives a straw-yellow solution in conc. sulfuric acid.

Subs. (air-dry), 0.4068: loss, 0.0297 *in vacuo* at 80° over H_2SO_4.
Calc. for $C_{30}H_{38}O_4N_3Cl.2.5H_2O$: H_2O, 7.70. Found: 7.30.
Subs. (anhydrous), 0.1229: 8.85 cc. N (29.5°, 759 mm.). Subs., 0.1507: 5.62 cc. $AgNO_3$ soln. (1 cc. = 0.00181 g. Cl).
Calc. for $C_{30}H_{38}O_4N_3Cl$: N, 7.79; Cl, 6.57. Found: N, 8.11; Cl, 6.75.

Hydroquinine 4-Chloroacetylamino-guaiacol, $C_{20}H_{26}O_2N_2.4,5\text{-}HO(CH_3O)C_6H_3NHCOCH_2Cl$.—In the case of 4-chloroacetylamino-guaiacol[1] the acetone was poured off from the gummy product and this taken up in a relatively large volume of boiling water and treated with boneblack. The practically colorless filtrate gradually darkened on standing, depositing the salt at the same time from the still warm solution as delicate, felted, almost colorless needles containing 2 molecules of water of crystallization. It is very difficultly soluble in cold water, fairly readily on boiling, and, judging by the low halogen figure obtained on analysis, appears to be somewhat hydrolyzed by this method of crystallization. An aqueous suspension gives a slowly developing orange color with ferric chloride. The anhydrous salt forms a brown jelly above 160°, becomes semifluid at about 190°, and is completely fluid and partially decomposed at 215°. It dissolves readily in methyl or ethyl alcohol or chloroform, and is difficultly soluble in boiling dry acetone, almost insoluble in boiling benzene. It dissolves in conc. sulfuric acid with a pale greenish yellow color.

[1] THIS JOURNAL, **41**, 1457 (1919).

Subs. (air-dry), 0.6924: loss, 0.0444 *in vacuo* at 80° over H_2SO_4.
Calc. for $C_{29}H_{36}O_5N_3Cl.2H_2O$: H_2O, 6.24. Found: 6.43.
Subs. (anhydrous), 0.1269: 8.6 cc. N (22.0°, 757 mm.). Subs., 0.1832: AgCl, 0.0415.
Calc. for $C_{29}H_{36}O_5N_3Cl$: N, 7.76; Cl, 6.55. Found: N, 7.81; Cl, 5.61.

Hydroquinine 3,4-Methylenedioxy-chloroacetanilide, $C_{20}H_{26}O_2N_2.3,4\text{-}CH_2O_2C_6H_3NHCOCH_2Cl$.—Starting with 2.2 g. of 3,4-methylenedioxy-chloroacetanilide[1] the reaction mixture deposited the crystalline salt on standing. Recrystallized from dry methyl ethyl ketone it separated as sheaves of long, delicate needles. These were warmed slightly and evacuated to remove adhering solvent and allowed to come to equilibrium in the air, retaining one molecule of water of crystallization or its equivalent. The yield was 1.5 g. The salt dissolves appreciably in cold water, quite readily on boiling, the solution forming a jelly on cooling and depositing a portion of the substance on standing as rosets of delicate needles. The anhydrous salt gradually melts, with slight preliminary softening, above 155–60°, turning orange and slowly evolving gas, and becoming completely fluid at 185°. It dissolves readily in methyl or ethyl alcohol or chloroform, less easily in dry acetone. It gives a lemon-yellow color with conc. sulfuric acid.

Subs. (air-dry), 0.5956: loss, 0.0196 *in vacuo* at 80° over H_2SO_4.
Calc. for $C_{29}H_{34}O_5N_3Cl.H_2O$: H_2O, 3.23. Found: 3.29.
Subs. (anhydrous), 0.1275: 8.6 cc. N (20.0°, 757 mm.). Subs., 0.1527: AgCl, 0.0398.
Calc. for $C_{29}H_{34}O_5N_3Cl$: N, 7.79; Cl, 6.57. Found: N, 7.83; Cl, 6.45.

Hydroquinine, 3,4-Dimethoxy-chloroacetanilide.—(From 2.3 g. of 3,4-dimethoxy-chloroacetanilide.[2]) The salt crystallized on standing and was dissolved in hot, dry acetone, filtered with the aid of boneblack, and the filtrate cautiously treated with ligroin, shaking to redissolve the gelatinous precipitate first formed. On seeding and letting stand overnight the salt separated as masses of hair-like needles and diamond-shaped plates containing solvent of crystallization. The yield was 3.1 g., an additional quantity, though less pure, separating from the mother liquors on standing. The anhydrous salt gradually softens and melts above 130° with gas evolution, becoming

[1] This Journal, **41**, 1457 (1919).
[2] *Ibid.*, **41**, 1461 (1919).

transparent at about 175° and completely fluid with decomposition at 205–10°. It softens under and dissolves readily in dry methyl or ethyl alcohol, dry chloroform, less readily, but freely in dry acetone. It is difficultly soluble in cold water, readily on heating, the solution gelatinizing on cooling. It dissolves with a yellow color in dil. hydrochloric or conc. sulfuric acid.

Subs. (anhydrous), 0.1347: 9.4 cc. N (21.0°, 765 mm.). Subs., 0.1237: 4.25 cc. $AgNO_3$ (1 cc. = 0.001794 g. Cl).
Calc. for $C_{30}H_{38}O_5N_3Cl$: N, 7.57; Cl, 6.38. Found: N, 8.16; Cl, 6.17.

Hydroquinine m-Chloroacetylamino-benzenesulfonamide, $C_{20}H_{26}O_2N_2.ClCH_2CONHC_6H_4SO_2NH_2(m\text{-})$. — Using *m*-chloroacetylamino - benzenesulfonamide[1] the reaction mixture deposited a portion of the quaternary salt as a gum. This was dissolved by adding ½ volume of absolute alcohol, after which the salt soon began to separate. Recrystallized from dry methyl alcohol it forms faintly pinkish, glistening, hexagonal platelets which gradually soften to a jelly above 180°, darkening, and finally decomposing slowly at 200–5°. Aqueous suspensions dissolve on boiling or on adding dil. acid or alkali. The salt is difficultly soluble in cold dry methyl or ethyl alcohol, more easily on boiling, and is also somewhat soluble in boiling dry acetone and practically insoluble in boiling dry chloroform.

Subs. (anhydrous), 0.0926: 8.0 cc. N (26.0°, 770 mm.). Subs., 0.1961: AgCl, 0.0468.
Calc. for $C_{28}H_{35}O_5N_4ClS$: N, 9.75; Cl, 6.17. Found: N, 9.99; Cl, 5.90.

(*F*) *Quaternary Salts of Hydroquinidine.*

Hydroquinidine Chloroacetamide.—Hydroquinidine iodoacetamide was prepared in almost quantitative yield by suspending the finely pulverized base in hot acetone and adding one equivalent of iodoacetamide in small portions, the alkaloid going into solution as the reaction proceeded. After boiling for a few moments the salt began to separate as glistening hexagonal plates and was converted into the chloride as were its previously discussed isomers and analogs. The chloride was salted out from the aqueous solution and recrystallized from a

[1] This Journal, 39, 2429 (1917).

small volume of water, forming aggregates of minute leaflets containing 5 molecules of water of crystallization and dissolving readily in water at room temperature, rather sparingly at 0°. The yield was about $\frac{1}{2}$ the amount of quaternary iodide used. The anhydrous salt gradually softens up to 185° to a yellow jelly which becomes completely fluid and decomposes at 210°. $[\alpha]_D^{25} = +179.9°$ in water, $c = 1.078$. It dissolves readily at room temperature in methyl or ethyl alcohol, chloroform, or acetone, and less easily in benzene.

Subs. (air-dry), 0.8437: loss, 0.1443 *in vacuo* at room temp., then at 80° over H_2SO_4.
Calc. for $C_{22}H_{30}O_3N_3Cl.5H_2O$: H_2O, 17.67. Found: 17.11.
Subs. (anhydrous), 0.1305: 11.5 cc. N (29.0°, 763 mm.). Subs., 0.1572: 7.16 cc. $AgNO_3$ soln. (1 cc. = 0.00181 g. Cl).
Calc. for $C_{22}H_{30}O_3N_3Cl$: N, 10.02; Cl, 8.45. Found: N, 10.0; Cl, 8.25.

Hydroquinidine p-Chloroacetylamino-phenol.—A suspension of the components in dry acetone soon cleared on boiling and after $\frac{1}{2}$ hour the salt suddenly crystallized and the mixture was allowed to stand in a warm place for 4 hours. The yield was 83% of the theoretical amount. Recrystallized by dissolving in hot absolute alcohol, adding about 3 volumes of dry ether, and seeding, the salt forms rosets of delicate needles which darken and soften slightly when heated and melt only when kept in the bath at 285° for a few moments. It is very difficultly soluble in boiling water and practically insoluble in dry chloroform or acetone, but dissolves at room temperature in absolute alcohol, more easily in dry methyl alcohol.

Subs., 0.1129: 8.4 cc. N (27.0°, 758 mm.). Subs., 0.1668: AgCl, 0.0460.
Calc. for $C_{28}H_{34}O_4N_3Cl$: N, 8.21; Cl, 6.93. Found: N, 8.45; Cl, 6.82.

The Hydrochloride.—The quaternary salt, rubbed under a small volume of 10% hydrochloric acid, formed a gummy mass which crystallized on standing. A solution of the salt in hot water containing a few drops of dil. hydrochloric acid was cooled and treated with the conc. acid until the initial turbidity barely redissolved on stirring. On seeding and letting stand in the cold the hydrochloride separated as faintly yellow, minute rhombs which deliquesced rapidly in the warm, moist room. The dried salt does not have this objectionable property. It melts and decomposes at 245–50° with preliminary darkening and

dissolves rather sparingly in cold water. It dissolves in dry methyl alcohol, sparingly in absolute alcohol, and is almost insoluble in boiling dry chloroform or acetone.

Subs., 0.2039: AgCl, 0.1050.
Calc. for $C_{28}H_{34}O_4N_3Cl.HCl$: Cl, 12.93. Found: 12.74.

Hydroquinidine Chloroacetyl-p-anisidine.—This salt was prepared in the same way and in the same yield as hydroquinidine-*p*-chloroacetyl-amino-phenol. Recrystallized from 25% alcohol it forms rosets of flat, cream-colored prisms which melt and decompose at 260–5°. It dissolves in dry chloroform and appreciably in methyl alcohol. It is sparingly soluble in absolute alcohol, more easily on boiling, and only very difficultly soluble in boiling water or dry acetone. It is optically inactive in dry chloroform, where $c = 1.019$.

Subs., 0.1110: 8.0 cc. N (26.0°, 765 mm.). Subs., 0.1209: 4.5 cc. $AgNO_3$ soln. (1 cc. = 0.00181 g. Cl).
Calc. for $C_{29}H_{36}O_4N_3Cl$: N, 7.99; Cl, 6.75. Found: N, 8.28; Cl, 6.74.

The Hydrochloride.—3 g. of the quaternary salt were dissolved in a few cc. of conc. hydrochloric acid and the solution then treated with small amounts of dry acetone, with shaking, until the volume was about 50 cc. On letting stand and rubbing occasionally the salt crystallized as faintly yellow, nacreous, rounded scales, crystallization being facilitated by the addition of more dry acetone and ether. The dried product, when rapidly heated to 195°, then slowly, melts and decomposes at 196–200°. It dissolves readily in water, methyl or ethyl alcohol, less easily in dry chloroform, and very difficultly in dry acetone.

Subs., 0.1148: 7.64 cc. $AgNO_3$ soln. (1 cc. = 0.00181 g. Cl).
Calc. for $C_{29}H_{36}O_4N_3Cl.HCl$: Cl, 12.61. Found: 12.05.

(*G*) *Quaternary Salts of Hydrocupreine.*

Hydrocupreine Chloroacetanilide Hydrochloride.—As hydrocupreine showed only very little tendency to combine with chloroacetanilide in boiling dry acetone it was found necessary to use iodoacetanilide, combining the substances in hot alcoholic solution. As the iodide showed no tendency to crystallize the solution was diluted until just turbid and shaken with freshly precipitated silver chloride. Most of the

alcohol was removed from the filtrate by concentration *in vacuo* and after dilution with hot water and treating with boneblack the clear yellow filtrate was treated with saturated sodium chloride solution, precipitating the chloride as an amorphous, yellow solid. This was collected and rubbed up with conc. hydrochloric acid, changing to a faintly yellow mass of spherules of microscopic needles, which were filtered off after dilution with a little more than an equal volume of water. When rapidly heated to 210°, then slowly, it melts and decomposes at 213–4°. It dissolves readily in dry methyl alcohol, less easily in cold water or absolute alcohol, and is almost insoluble in dry chloroform or acetone.

Subs., 0.1428: 10.0 cc. N (21.5°, 759 mm.). Subs., 0.1566: AgCl, 0.0888.
Calc. for $C_{27}H_{32}O_3N_3Cl.HCl$: N, 8.11; Cl, 13.68. Found: N, 8.11; Cl, 14.03.

Hydrocupreine Chloroacetyl-p-anisidine Hydrochloride.—3.3 g. of hydrocupreine, 2 g. of anhydrous sodium iodide, and 2 g. of chloroacetyl-*p*-anisidine were boiled for one hour in dry acetone. The base quickly dissolved and was replaced by a heavy precipitate of sodium chloride. The filtrate and washings were diluted with water and shaken for 45 minutes with freshly precipitated silver chloride. After adding an excess of hydrochloric acid to the filtrate it was concentrated to small bulk *in vacuo*, the last portions of the salt separating as colorless, hair-like needles. After washing with a little 10% hydrochloric acid the residue was dried *in vacuo*, dissolved in boiling absolute alcohol containing a little dry hydrochloric acid, cooled, and seeded with crystals obtained by rubbing a portion of the crude salt with absolute alcohol. The pure salt forms radiating masses of minute, pale yellow needles, which, after drying as usual melt to an orange jelly at 203–5.5° and become completely fluid, with gas evolution and darkening, at about 210°. It dissolves easily in water or methyl alcohol, slowly but freely in dry chloroform, and is appreciably soluble in absolute alcohol at room temperature, readily on warming. An aqueous solution gives a pale brownish color with ferric chloride.

Subs., 0.1205: 8.4 cc. N (24.5°, 750 mm.). Subs., 0.1088; 7.8 cc. $AgNO_3$ soln. (1 cc. = 0.001794 g. Cl).
Calc. for $C_{28}H_{34}O_4N_3Cl.HCl$: N, 7.57; Cl, 12.93. Found: N, 7.89; Cl, 12.86.

(*H*) *Quaternary Salts of Ethylhydrocupreine.*

Ethylhydrocupreine Methochloride.—The methiodide[1] in aqueous suspension, was quantitatively converted into the chloride in the usual manner. Recrystallized from water it forms aggregates of silky needles containing 2.5 molecules of water of crystallization and is rather difficultly soluble in cold water. The anhydrous salt softens when heated, melting to a paste at about 160°, then turning yellow and finally melting completely to an orange liquid filled with bubbles at 198–9°. $[\alpha]_D^{22} = -176.7°$ in water, $c = 0.651$. It dissolves readily in dry methyl or ethyl alcohol or dry chloroform, less easily in dry acetone.

Subs. (air-dry), 0.6166: loss, 0.0633 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{22}H_{31}O_2N_2Cl.2.5H_2O$: H_2O, 10.33. Found: 10.27.
Subs. (anhydrous), 0.2442: AgCl, 0.0902.
Calc. for $C_{22}H_{31}O_2N_2Cl$: Cl, 9.07. Found: 9.14.

Ethylhydrocupreine Iodoacetamide.—Ethylhydrocupreine and iodoacetamide reacted quickly in acetone solution. Most of the acetone was evaporated off and the residue dissolved in hot water, the iodide separating in almost quantitative yield on cooling and rubbing. Recrystallized twice from water, using boneblack, it forms rosets of needles and long, narrow plates containing 3 molecules of water of crystallization. The anhydrous substance softens to a jelly at 140–50°, gradually liquefying and darkening, and becoming completely fluid at about 185°. $[\alpha]_D^{25} = -115.4°$ in absolute alcohol, $c = 0.910$. It dissolves readily in methyl or ethyl alcohol, chloroform, or acetone.

Subs. (air-dry), 0.5403: loss, 0.0501 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{23}H_{32}N_3I.3H_2O$: $H_2O = 9.33$. Found: 9.27.
Subs. (anhydrous), 0.1611: AgI, 0.0709.
Calc. for $C_{23}H_{32}O_3N_3I$: I, 24.17. Found: 23.78.

Ethylhydrocupreine Chloroacetamide.—The iodide was converted into the chloride and salted out with sodium chloride. Recrystallized from water it forms faintly yellow aggregates of long, narrow plates containing a little over 3 molecules of water of crystallization and dissolving fairly readily in water. The anhydrous salt melts to a jelly

[1] This Journal, **41**, 825 (1919).

at 145–60°, turns yellow, then orange, and becomes completely fluid at about 195°. $[\alpha]_D^{23} = -114.8°$ in water, $c = 1.124$. It dissolves readily in absolute alcohol.

Subs. (air-dry), 0.6813: loss, 0.0815 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{23}H_{32}O_3N_3Cl.3H_2O$: H_2O, 11.08. Found: 11.97.
Subs. (anhydrous), 0.0852: 7.3 cc. N (27.0°, 753 mm.). Subs., 0.1705: 7.68 cc. $AgNO_3$ soln. (1 cc. = 0.00181 g. Cl).
Calc. for $C_{23}H_{32}O_3N_3Cl$: N, 9.69; Cl, 8.17. Found: N, 9.66; Cl, 8.16.

Ethylhydrocupreine p-Chloroacetylamino-phenol.—The salt, prepared by the direct method, crystallized in good yield on letting stand. Recrystallized from dry methyl ethyl ketone it formed rosets of delicate needles which came to equilibrium in the air with one molecule of water of crystallization. When rapidly heated to 175°, then slowly, it melted at 178–82° to a jelly. $[\alpha]_D^{22} = -71.8°$ in absolute alcohol, $c = 0.844$. The salt dissolves readily in absolute alcohol and very difficultly in water or dry acetone. It softens under dry chloroform and dissolves to some extent.

Subs. (air-dry), 0.5319: loss, 0.0181 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{29}H_{36}O_4N_3Cl.H_2O$: H_2O, 3.31. Found: 3.40.
Subs. (anhydrous), 0.1359: 9.4 cc. N (18.0°, 760 mm.).
Calc. for $C_{29}H_{36}O_4N_3Cl$: N, 7.99. Found: 8.12.

The Hydrochloride.—On rubbing with 1:1 hydrochloric acid the quaternary salt changed to a pasty mass which gradually crystallized. Its solution in hot water containing a little hydrochloric acid was cooled, treated with the concentrated acid until the initial turbidity barely redissolved, and seeded. The hydrochloride separated in the refrigerator as almost colorless rosets of delicate needles. The anhydrous salt darkens at about 185°, gradually melting to an orange liquid and evolving gas at 196–7°. It dissolves readily in water, methyl or ethyl alcohol, and is practically insoluble in dry acetone.

Subs., 0.1528: 10.62 cc. $AgNO_3$ soln. (1 cc. = 0.00181 g. Cl).
Calc. for $C_{29}H_{36}O_4N_3Cl.HCl$: Cl, 12.61. Found: 12.58.

Ethylhydrocupreine Chloroacetyl-p-anisidine.—The salt was obtained crystalline by evaporation of the acetone solution. After recrystallization from methyl ethyl ketone the yield was 82.5% of the theory. Recrystallized from boiling water, in which it is only difficultly soluble, it

separates as delicate, hair-like needles containing 1.5 molecules of water of crystallization. The anhydrous salt softens to a jelly at 145–55°, turning yellow and liquefying completely by the time 200° is reached. $[\alpha]_D^{25} = -69.0°$ in absolute alcohol, $c = 1.574$. It dissolves readily in methyl or ethyl alcohol or chloroform, more slowly in dry acetone. It gives a pale yellow color in conc. sulfuric acid.

Subs. (air-dry), 0.7569: loss, 0.0334 *in vacuo* at 80°, then 100° over H_2SO_4.
Calc. for $C_{30}H_{38}O_4N_3Cl.1.5H_2O$: H_2O, 4.41. Found: 4.77.
Subs. (anhydrous), 0.1533: 10.6 cc. N (25.0°, 767 mm.). Subs., 0.1188: 4.3 cc. $AgNO_3$ soln. (1 cc. = 0.00181 g. Cl).
Calc. for $C_{30}H_{38}O_4N_3Cl$: N, 7.79; Cl, 6.57. Found: N, 7.99; Cl, 6.56.

The Hydrochloride.—A solution of the quaternary salt in an excess of absolute alcoholic hydrochloric acid was concentrated to dryness *in vacuo* and the gummy residue digested in the flask with dry acetone, a portion dissolving. After several days the hydrochloride began to crystallize as prismatic needles and became entirely crystalline on rubbing and letting stand. When rapidly heated to 200°, then slowly, the dried salt darkens and sinters at 201–4° and melts and decomposes at 204–5°. It dissolves readily in methyl or ethyl alcohol and only incompletely in water, owing to hydrolysis.

Subs., 0.1374: 9.46 cc. $AgNO_3$ soln. (1 cc. = 0.00181 g. Cl).
Calc. for $C_{30}H_{38}O_4N_3Cl.HCl$: Cl, 12.30. Found: 12.47.

Ethylhydrocupreine Chloroacetyl-p-phenetidine.—Ligroin was added to the reaction mixture and the solution allowed to stand in the ice box with occasional rubbing, the salt crystallizing in a yield of 80% of the theoretical amount. Recrystallized with the aid of boneblack from boiling water, in which it is sparingly soluble it separated on seeding as woolly masses of delicate needles containing 1.5 molecules of water of crystallization. The anhydrous salt softens to a jelly above 130°, gradually liquefying and turning yellow as the temperature is raised, until completely fluid, with slow decomposition at about 210°. $[\alpha]_D^{25.5} = -75.25°$ in 95% alcohol, $c = 0.917$. It dissolves extremely easily in chloroform, very readily on methyl or ethyl alcohol, and easily in dry acetone.

Subs. (air-dry), 0.5089: loss, 0.0232 *in vacuo* at 80°, then 100° over H_2SO_4.
Calc. for $C_{31}H_{40}O_4N_3Cl.1.5H_2O$: H_2O, 4.65. Found: 4.56.

Subs. (anhydrous), 0.1198: 8.25 cc. N (27.0°, 762 mm.). Subs., 0.1276: 4.47 cc. $AgNO_3$ soln. (1 cc. = 0.00181 g. Cl).
Calc. for $C_{31}H_{40}O_4N_3Cl$: N, 7.59; Cl, 6.40. Found: N, 7.86; Cl, 6.35.

The Hydrochloride.—This salt was obtained in exactly the same way as the corresponding anisidine compound, forming aggregates of minute, thick plates with tapering ends. It begins to turn yellow above 150°, darkens at about 200°, then softens and sinters, and melts and decomposes at 208°. It softens under water and dissolves slowly but completely on shaking. It is also readily soluble in absolute alcohol or chloroform and only very sparingly in dry acetone.

Subs., 0.1357: 8.7 cc. N (25.0°, 750 mm.). Subs., 0.1415: 9.42 cc. $AgNO_3$ soln. (1 cc. = 0.00181 g. Cl).
Calc. for $C_{31}H_{40}O_4N_3Cl.HCl$: N, 7.12; Cl, 12.02. Found: N, 7.25; Cl, 12.05.

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SYNTHESES IN THE CINCHONA SERIES. III.

Azo Dyes Derived from Hydrocupreine and Hydrocupreidine.

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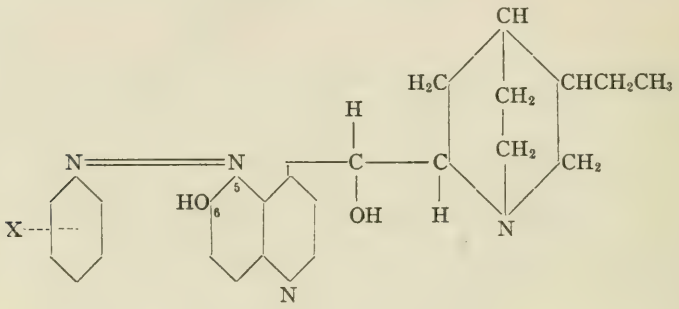
The subject matter of this and the previous paper (p. 297) was presented at the June meeting of the New York Section of the American Chemical Society. Three months later, while the present material was being prepared for publication we received the paper of Giemsa and Halberkann[1] on "Azo-5 and Amino-5 Compounds of Cupreine, Hydrocupreine and their Methyl and Ethyl Ethers," in which the idea of azo-cinchona compounds is elaborated, although only one of the substances described in the present paper (*p*-sulfophenylazo-hydrocupreine) was studied. The work which we now report has been in progress for over two years and represents but a portion of the studies which we are making in the field of the cinchona alkaloids.[2] Among our objects in studying the azo derivatives of the hydroxy alkaloids was the reduction of suitable members to the amino alkaloids, which we now see has also been accomplished by the German workers. As far as 5-aminohydrocupreine is concerned, we, too, prepared this interesting compound almost two years ago, and since our work with it and analogous substances is still in progress, we shall reserve our discussion of the amino alkaloids for a future paper.

We have found that hydrocupreine and hydrocupreidine behave in a normal manner as phenolic compounds and yield well-defined azo dyes with diazotized aromatic amines.

[1] *Ber.*, **52**, 906 (1919).

[2] For Paper I of this series, see This Journal, **41**, 818 (1919).

We have assumed from the first that position 5 (*ana*) in the quinoline nucleus is the place of entrance of the azo group



since this is the only available position which would satisfy the usual rules of substitution for a 6-hydroxyquinoline derivative, a conclusion also arrived at by Giemsa and Halberkann.

Of the dyes prepared by us from a large variety of aromatic amines the majority crystallized without difficulty or formed crystalline salts, and while others could not be made to crystallize we are convinced that methods for their crystallization could have been found in a more detailed and time-consuming study than we were able to give. In general, the phenyl and tolyl dyes, as well as those with *m*-substituents on the benzene ring gave orange to orange-red solutions in dil. acids and dyed silk various shades of orange; *o*- and *p*-alkoxy groups deepened these colors toward the red, while the dyes from the naphthylamines dyed silk a deep rose color. As a class these substances had the property of dyeing unmordanted cotton, a property also shared by 5-phenylazo-6-hydroxyquinoline,[1] which was prepared for comparison.

While the crystalline dyes which did not contain negative salt-forming groups on the aromatic amine were generally only slightly soluble in cold *N* aqueous alkali and only incompletely on boiling, solutions of the dyes in a little alcohol gave clear, deep red to purplish red solutions on dilution with *N* sodium hydroxide. All of the dyes, of course, were soluble in dil. acids.

Many of the substances described in this paper were highly bactericidal *in vitro*, a property which will be discussed in the appropriate place by our colleague, Dr. Martha Wollstein.

[1] Mathëus, *Ber.*, **21**, 1642 (1888).

EXPERIMENTAL.

Since the azo compounds to be described in the following pages were all prepared by essentially the same technique this will be given at the start, and in cases in which modifications proved necessary these will be described under the individual compounds.

The aromatic amine was diazotized as usual in dil. hydrochloric acid solution containing 4 equivalents of the acid. The solution was then slowly added to a turbined solution of hydrocupreine in dil. sodium hydroxide, the quantity of the latter being 6 equivalents, or 2 in excess of the amount of hydrochloric acid originally employed, this having been chosen in order to hold the alkaloid in solution during the coupling. The volume of the alkaline solution was from 100 to 150 cc. for 3.1 g. (0.01 mol.) of the base and the temperature during the coupling was kept at 10–15°. As a rule the coupling proceeded rapidly, the dye in most cases separating during the reaction as a flocculent precipitate. In most instances precipitation was completed by the final addition of ammonium chloride solution. After filtering off and washing, the dye was purified by grinding under very dilute hydrochloric acid, filtering from insoluble material, and reprecipitating from the diluted filtrate with ammonia.

(*A*) *Dyes Derived from Hydrocupreine.*

Phenylazo-hydrocupreine.—On adding insufficient ammonia for neutralization to the dil. hydrochloric acid solution of the crude product a tarry precipitate which separated was filtered off before making alkaline with ammonia. The dye was obtained in slightly greater yield than the amount of hydrocupreine used and forms an orange-red, amorphous powder which is readily soluble in the usual organic solvents. It gradually softens above 85°, melting completely at about 130°. It is soluble in dil. acids with an orange color and although insoluble in too dilute sodium hydroxide it dissolves in 2% alkali with a cherry-red color. It gives a deep orange-brown solution in conc. sulfuric acid.

Subs., 0.1364: 15.3 cc. N (19.0°, 754 mm.).
Calc. for $C_{25}H_{28}O_2N_4$: N, 13.46. Found: 13.02.

Phenylazo-hydrocupreine Dihydrochloride.—The purified base was dissolved in a small volume of 10% hydrochloric acid, chilled, and seeded with crystals obtained by dissolving a test portion in 1:1 hydrochloric acid and adding saturated salt solution. After standing in the ice box, the salt was recrystallized from a small volume of 10% hydrochloric acid and let stand at 0°, separating as dark red, felted needles containing 4 molecules of water of crystallization. When rapidly heated to 180°, then slowly, the anhydrous salt decomposes at 181–3° with preliminary darkening and sintering, and dissolves readily in the cold in water, alcohol, methyl alcohol, or chloroform. It is almost insoluble in dry ether or hot benzene and only sparingly soluble in hot dry acetone. An aqueous solution is readily decolorized by stannous chloride with the formation of aniline hydrochloride and the hydrochloride of aminohydrocupreine.

For analysis the salt was dried *in vacuo* first at room temperature over sulfuric acid and crushed alkali and finally at 80° over sulfuric acid.

Subs., air-dry, 0.5910: loss, 0.0768.
Calc. for $C_{25}H_{28}O_2N_4.2HCl.4H_2O$: H_2O, 12.84. Found: 12.99.
Subs., anhydrous, 0.1325: 13.0 cc. N (23.0°, 760 mm.). Subs., 0.1038: AgCl, 0.0578.
Calc. for $C_{25}H_{28}O_2N_4.2HCl$: N, 11.45; Cl, 14.49. Found: N, 11.32; Cl, 13.78.

o-Tolylazo-hydrocupreine.—The purified amorphous dye was dissolved in a small volume of hot absolute alcohol, chilled, and seeded with crystals formed by the spontaneous evaporation of an alcoholic solution of a test portion. The dye slowly crystallized as deep red, spherular masses of microscopic crystals which darken and sinter above 100°, melting to a tar from 110 to 30°. It is readily soluble in boiling methyl or ethyl alcohol, sparingly at 0°, and dissolves very easily in chloroform or benzene, less readily in dry acetone. It dissolves in conc. sulfuric acid with an orange-red color and partially in hot 5% sodium hydroxide, yielding a cherry-red solution. In dilute acids the color is orange-red.

Subs., 0.1413: 16.2 cc. N (24.0°, 758.5 mm.).
Calc. for $C_{26}H_{30}O_2N_4$: N, 13.02. Found: 13.16.

m-Tolylazo-hydrocupreine.—The purified base was obtained in good yield as an orange-red, amorphous powder which is readily soluble in the usual neutral organic solvents, and is quite soluble in cold *N* sodium hydroxide and practically completely, on boiling, forming a dull red solution. It gradually melts above 103° with preliminary sintering, becoming completely fluid at 125–30°.

Subs., 0.1119: 12.6 cc. N (27.0°, 765 mm.).
Calc. for $C_{26}H_{30}O_2N_4$: N, 13.02. Found: 12.89.

The Dihydrochloride.—The base was rubbed under sufficient conc. hydrochloric acid to give a reaction strongly acid to congo red paper and the solution diluted with dry acetone. On adding dry ether until a slight permanent turbidity formed the salt gradually separated on standing and rubbing as aggregates of red, microscopic crystals. After air-drying, the salt contained approximately 1.5 molecules of water of crystallization. When anhydrous it darkens and softens above 160° and decomposes at about 195°. It is very easily soluble in dry methyl alcohol, less easily in absolute alcohol. It dissolves with difficulty in dry acetone or chloroform but is quite soluble in the latter on boiling. It is readily soluble in water with a reddish orange color.

Subs., 0.6548: loss, 0.0373 *in vacuo* at room temp. over H_2SO_4 + NaOH.
Calc. for $C_{26}H_{30}O_2N_4.2HCl.1.5H_2O$: H_2O, 5.70. Found: 5.09.
Subs., anhydrous, 0.1034: 10.1 cc. N (26.0°, 757 mm.). Subs., 0.1442: AgCl, 0.0832.
Calc. for $C_{26}H_{30}O_2N_4.2HCl$: N, 11.14; Cl, 14.09. Found: N, 11.11; Cl, 14.27.

p-Tolylazo-hydrocupreine.—The crude dye was made to crystallize by warming with 85% alcohol, the separation being completed after cooling by the cautious addition of about $\frac{1}{3}$ volume of water. Recrystallized from amyl alcohol, in which it dissolved readily at the boiling point and sparingly in the cold, it forms glistening, orange-red leaflets. When rapidly heated to 200°, then slowly, the dye melts at 202–5° with gas evolution and preliminary softening. It is rather sparingly soluble in the cold in absolute methyl or ethyl alcohol or acetone, but quite readily on warming, and also dissolves easily in chloroform or toluene. The solution in conc. sulfuric acid is bright red, and orange-red in dil. acids.

Subs., 0.1511: 17.2 cc. N (26.5°, 752 mm.).
Calc. for $C_{26}H_{30}O_2N_4$: N, 13.02. Found: 12.84.

α-Naphthylazo-hydrocupreine.—On dissolving the purified dye in dry acetone it separated almost immediately as glistening, copper-colored, diamond-shaped platelets. It separates from alcohol as deep maroon plates with a bronzy luster, which crush to a dark, purplish red powder and melt slowly to a tar at 146–9° with preliminary sintering. It is soluble in hot alcohol or acetone, more so in the cold in methyl alcohol or benzene, very readily in chloroform, and only sparingly in dry ether and in hot dil. sodium hydroxide. It yields a bright red solution in dil. hydrochloric acid and a deep blue color in conc. sulfuric acid.

Subs., 0.1232: 13.0 cc. N (20.5°, 756 mm.).
Calc. for $C_{29}H_{30}O_2N_4$: N, 12.02. Found: 12.21.

β-Naphthylazo-hydrocupreine.—In the purification of the crude dye it was found necessary to use warm dil. hydrochloric acid, as the hydrochloride tended to separate from cold solutions in gelatinous form. Recrystallized from amyl alcohol, in which it is soluble only near the boiling point, the base separates as glistening, woolly, scarlet needles which decompose at 240–4°. The solution in dil. hydrochloric acid is bright orange-red, while that in sulfuric acid is deep violet. It is soluble in chloroform but only very difficultly soluble in alcohol, dry acetone, or benzene, and is insoluble in hot dil. sodium hydroxide solution.

Subs., 0.1037: 11.0 cc. N (21.5°, 754 mm.).
Calc. for $C_{29}H_{30}O_2N_4$: N, 12.02. Found: 12.20.

Diphenyl-p-bisazo-hydrocupreine.—1.4 g. of benzidine were dissolved in hot water containing 13 g. of conc. hydrochloric acid, chilled, and diazotized with 1.05 g. of sodium nitrite. The solution was coupled with 4.5 g. of hydrocupreine dissolved in 180 cc. of *N* sodium hydroxide diluted to 300 cc. The resulting dye was purified in the usual manner, yielding 4.5 g. of an amorphous product. When dissolved in pyridine, filtered, and cautiously treated with water the dye precipitated as a dubiously crystalline, purplish brown powder. It melts to a tar, with preliminary sintering, above 204° and decomposes at about 220°. The dye is easily soluble in chloroform, sparingly in

absolute alcohol or benzene, and very difficultly in dry ether. It dissolves in dil. acids with a deep, purplish red color and only partially in boiling *N* sodium hydroxide solution with a dull red color. The solution in conc. sulfuric acid is an intense violet-blue.

Subs., 0.1178: 13.3 cc. N (28.0°, 758 mm.).
Calc. for $C_{50}H_{54}O_4N_8$: N, 13.49. Found: 12.77.

m-Nitrophenylazo-hydrocupreine.—Modification of the usual procedure was necessary in this case, as otherwise the yield was very poor. 2.1 g. of *m*-nitraniline were dissolved by heating with 12.7 g. of conc. hydrochloric acid and about an equal volume of water, rapidly chilled to form a paste of small crystals of the hydrochloride, and diazotized below 10° with a conc. sodium nitrite solution. A solution of 4.5 g. of hydrocupreine was prepared with 200 cc. of *N* sodium hydroxide, diluting to 300 cc. and cautiously adding 14.3 g. of sodium bicarbonate (enough to combine with all but 2 equivalents of the free alkali), stirring until clear, and chilling to 10–15°. After slowly adding the diazo solution and stirring for 15 minutes, the orange dye was filtered off and purified in the usual way, a relatively large amount of acid-insoluble by-products remaining on the filter. The purified product was dissolved in boiling alcohol and separated on cooling and rubbing. Recrystallized from alcohol it forms aggregates of red platelets with an orange luster, which melt at 150.5–1.5° to a turbid liquid and yield an orange-red powder when crushed. It dissolves in conc. sulfuric acid or in dil. acids with a red-orange color and gives a deep red solution in boiling *N* sodium hydroxide. It is quite readily soluble in boiling absolute methyl or ethyl alcohol, somewhat sparingly in the cold, but dissolves more easily in cold acetone, benzene, or chloroform.

Subs., 0.1164: 15.3 cc. N (26.0°, 762 mm.).
Calc. for $C_{25}H_{27}O_4N_5$: N, 15.19. Found: 15.05.

p-Nitrophenylazo-hydrocupreine.—A diazotized *p*-nitraniline solution was slowly added to a solution of hydrocupreine prepared in exactly the same way as in the preceding case. The brown-orange precipitate of the dye was washed with water, sucked as dry as possible, and added to boiling alcohol, a portion beginning to crystallize

even from the boiling solution. Recrystallized from butyl alcohol it forms brown-red, glistening plates and flat prisms which appear orange under the microscope and crush to an orange-red powder. The substance gradually darkens and softens above 200° and melts and decomposes at about 220°. It dissolves in conc. sulfuric acid or in dil. acids with a red-orange color and gives a deep purple solution in boiling *N* sodium hydroxide. It is rather sparingly soluble even in boiling absolute alcohol or dry acetone, somewhat more easily in boiling methyl alcohol, and is quite soluble in boiling benzene, difficultly in the cold. Cold chloroform dissolves it very readily.

Subs., 0.1218: 16.3 cc. N (25.5°, 761 mm.).
Calc. for $C_{25}H_{27}O_4N_5$: N, 15.19. Found: 15.32.

m-Acetaminophenylazo-hydrocupreine.—In the case of *m*-aminoacetanilide hydrochloride[1] after coupling was complete the dye was thrown out from the deep purple solution by the addition of ammonium chloride. As it showed a tendency to run through the filter when washed with water, a little ammonia was added to the wash water, inhibiting this tendency. The purified substance forms a deep maroon, amorphous powder with a greenish reflex which slowly melts from 155 to 80° after preliminary darkening and sintering. It gives a brown solution in conc. sulfuric acid appearing red-brown by transmitted light, and a deep red color in dil. hydrochloric acid. It melts under dry methyl or ethyl alcohol and dissolves partially, forming deep red solutions. It is rather sparingly soluble in dry acetone but dissolves readily in chloroform. The color in dil. sodium hydroxide solution is a deep purplish red.

Subs., 0.1048: 13.8 cc. N (25.0°, 758 mm.).
Calc. for $C_{27}H_{31}O_3N_5$: N, 14.80. Found: 15.04.

m-Uraminophenylazo-hydrocupreine.—*m*-Aminophenylurea hydrochloride[2] coupled almost at once, forming a deep purple solution from which the dye was precipitated with ammonium chloride as orange flocks which tended to form a colloidal solution on washing with water. Addition of a few drops of ammonia to the wash water diminished this

[1] This Journal, **39**, 1448 (1917).
[2] *Ibid.*, **39**, 1449 (1917).

tendency. The purified dye forms an amorphous powder which darkens and sinters above 140° and gradually melts, swells, and decomposes at 165–85°. It dissolves in dil. hydrochloric acid with an orange-red color and in dil. alkali giving a cherry-red solution. The solution in conc. sulfuric acid is a deep brown-orange. The dye melts under dry methyl or ethyl alcohol and then dissolves, more easily in the former. It is less soluble in dry acetone or chloroform and only very slightly in boiling water.

Subs., 0.1077: 17.2 cc. N (25.0°, 753 mm.).
Calc. for $C_{26}H_{30}O_3N_6$: N, 17.72. Found: 18.13.

p-Aminophenylazo-hydrocupreine.—The acetamino compound (see below) was boiled for one hour with 5 parts of 1:1 hydrochloric acid and the solution diluted, filtered, and made alkaline with ammonia. Reprecipitated with ammonia from its solution in very dil. hydrochloric acid, it separates as purplish brown, amorphous flocks which melt at 155–65° with preliminary softening. It is slightly soluble in cold water with a purplish color, more easily in boiling water with a brownish red color, and yields an orange-brown solution in conc. sulfuric acid. An aqueous suspension dissolves with a deep red color on adding a drop of acetic acid, changing to orange-brown with excess of hydrochloric acid. It dissolves readily in methyl or ethyl alcohol, acetone, or chloroform, less easily in cold benzene.

Subs., 0.1152: 15.6 cc. N (22.5°, 765 mm.).
Calc. for $C_{25}H_{29}O_2N_5$: N, 16.24. Found: 15.75.

p-Acetaminophenylazo-hydrocupreine.—Precipitation of the dark red dye was completed by the addition of ammonium chloride. On diluting a methyl alcoholic solution of the purified, amorphous substance with water until the initial turbidity just redissolved, and seeding with crystals deposited on spontaneous evaporation of a test portion in methyl alcohol, the substance crystallized slowly as deep maroon, glistening, microscopic rhombs containing 1.5 molecules of water of crystallization, the separation being completed by the cautious addition from day to day of small amounts of hot water. The anhydrous substance melts to a tar at 158–62° with preliminary softening. It dissolves readily in methyl or ethyl alcohol or acetone, less easily in

benzene. In dil. sodium hydroxide it forms a purple-red solution and dissolves in conc. sulfuric acid with an orange-red color.

Subs., air-dry, 0.3677: loss, 0.0201 *in vacuo* at 80° over H_2SO_4.
Calc. for $C_{27}H_{31}O_3N_5.1.5H_2O$: H_2O, 5.40. Found: 5.47.
Subs., anhydrous, 0.1308: 17.4 cc. N (25.5°, 747 mm.).
Calc. for $C_{27}H_{31}O_3N_5$: N, 14.80. Found: 14.95.

p-Uraminophenylazo-hydrocupreine.—The purified dye from *p*-aminophenylurea[1] was dissolved in a rather large volume of 85% alcohol and cautiously diluted with water until the turbidity just redissolved. On standing and rubbing crystallization slowly started and was aided from time to time by the careful addition of water. The substance formed deep maroon, rhombic crystals accompanied by small amounts of amorphous material, but the amount obtained was too small for further purification. The anhydrous substance blackens and sinters above 170° and swells and chars at about 190°. It melts under absolute methyl or ethyl alcohol and dissolves partially, but is not very soluble even on boiling. It is sparingly soluble in hot, dry acetone or chloroform, and dissolves in conc. sulfuric acid with a deep red color. The solution in dil. alkali is purplish red and red in dil. acid.

Subs., 0.1276: 20.2 cc. N (26.0°, 756 mm.).
Calc. for $C_{26}H_{30}O_3N_6$: N, 17.72. Found: 17.99.

p - Hydroxyphenylazo - hydrocupreine.—*p* - Benzoyloxy-phenylazo-hydrocupreine dihydrochloride (see below) was dissolved in hot 95% alcohol and the solution chilled and treated with 25% sodium hydroxide solution, causing precipitation of a portion of the dye as a gum. This was redissolved in hot 95% alcohol, added to the main portion, and the whole made strongly alkaline with 25% sodium hydroxide. After about 10 minutes the dark, cherry-red solution was poured into water and treated with an excess of saturated ammonium chloride solution, precipitating the hydroxy dye, which was filtered off and dried. When starting with the quantities given below, the yield was 4.0 g. The crude dye was powdered and boiled with dry acetone, crystallizing before all was dissolved. After chilling and washing with a little cold, dry acetone the substance was recrystallized by

[1] This Journal, **39**, 1456 (1917).

dissolving in hot commercial butyl alcohol, cooling, adding an equal volume of dry ether, and then ligroin to incipient turbidity, filtering, seeding, and letting stand in the ice box. The dye separated as spherules of deep maroon, microscopic needles which may be pulverized to a dark brown-red powder and retain $\frac{1}{2}$ molecule of butyl alcohol of crystallization on drying *in vacuo* over calcium chloride and paraffin, the solvent being given off very slowly even at 100° *in vacuo* over sulfuric acid. The solvent-free substance gradually melts, blackens and swells at 150–60° and is readily soluble in dry methyl or ethyl alcohol with a brown-red color. It is less soluble in cold dry acetone, dissolving on boiling with a bright red color. It gives an intense red color in conc. sulfuric acid, appearing purplish in thin layers, and dissolves in dil. hydrochloric acid with a bright red, in dil. sodium hydroxide with a cherry-red color. A solution in warm 10% hydrochloric acid deposits the dihydrochloride on cooling as delicate, red needles.

Subs., dried at room temp., 0.3740: loss, 0.0304 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{25}H_{28}O_3N_4.0.5C_4H_9OH$: C_4H_9OH, 7.89. Found: 8.13.
Subs., solvent-free, 0.1265: 14.2 cc. N (24.0°, 761 mm.).
Calc. for $C_{25}H_{28}O_3N_4$: N, 12.96. Found: 12.92.

p-Benzoyloxy-phenylazo-hydrocupreine Dihydrochloride.—3.6 g. of *p*-aminophenyl benzoate were diazotized by suspending in a little water, adding 5.5 g. of conc. hydrochloric acid, and very slowly dropping in 3.3 cc. of 5 *N* sodium nitrite solution. The filtrate was slowly added to a solution of 4.7 g. of hydrocupreine in 120 cc. of *N* aqueous sodium hydroxide, to which 50 cc. of water and 7.7 g. of sodium bicarbonate (leaving 2 mols. of uncombined alkali) had been added with vigorous stirring. The dye, which separated rapidly, was filtered off, dissolved in dil. hydrochloric acid, the solution filtered, and treated with saturated salt solution until the initial turbidity just redissolved. On seeding with crystals obtained by precipitating a portion of the solution with sodium chloride and letting stand, the dihydrochloride separated rapidly and was washed with 10% hydrochloric acid, dissolved in 50% alcohol, and treated with an equal volume of the 10% acid. On seeding and chilling the dihydrochloride separated as minute, orange needles containing 2.5 molecules of water

of crystallization and dissolving quite freely in water with a red-orange color, leaving a trace of insoluble material. The anhydrous salt melts, blackens, and decomposes at about 185–95°, and dissolves readily in cold dry methyl alcohol, less easily in absolute alcohol. It is sparingly soluble in boiling dry acetone, more easily in dry chloroform. It dissolves in conc. sulfuric acid with an intense red color, appearing purplish in thin layers, the same colors being observed in the clear solution obtained by diluting an alcoholic solution with *N* aqueous sodium hydroxide.

Subs., air-dry, 0.6640: loss, 0.0483 *in vacuo* at room temp. over H_2SO_4 + NaOH.
Calc. for $C_{32}H_{32}O_4N_4.2HCl.2.5H_2O$: H_2O, 6.89. Found: 7.27.
Subs., anhydrous, 0.1239: 10.0 cc. N (28.0°, 759 mm.). Subs., 0.2259: AgCl, 0.0998.
Calc. for $C_{32}H_{32}O_4N_4.2HCl$: N, 9.20; Cl, 11.63. Found: N, 9.14: Cl, 10.93.

2-Methoxyphenylazo-hydrocupreine.—During the coupling the scarlet dye separated almost immediately. The purified substance forms a dark red, amorphous powder which darkens and softens above 105°, melts to a tar above 120°, and is completely fluid at about 160°. It is easily soluble in alcohol, acetone, or benzene, less readily in ether. It dissolves in conc. sulfuric acid with an intense purplish red color and in dil. acids with a redder shade than the phenyl or tolyl compounds. It is soluble in hot 5% sodium hydroxide solution with a cherry-red color.

Subs., 0.1076: 12.4 cc. N (30.0°, 752 mm.).
Calc. for $C_{26}H_{30}O_3N_4$: N, 12.55. Found: 12.85.

3-Methoxyphenylazo-hydrocupreine.—The crude dye from 2 g. of *m*-anisidine[1] was dried and ground in a mortar with about 25 cc. of commercial butyl alcohol. The substance dissolved and then quickly crystallized out in a yield of 3.9 g. Recrystallized from butyl alcohol it separates as dark red rhombs with a golden luster, crushing to an orange-red powder and melting at 188–90°, with slight gas evolution and preliminary softening. It dissolves only partially in boiling *N* sodium hydroxide solution, but if dissolved in a little alcohol and then diluted with *N* sodium hydroxide a clear, deep cherry-red solu-

[1] THIS JOURNAL, 41, 1452 (1919).

tion is obtained. The dye dissolves in dil. acids with an orange-red color and in conc. sulfuric acid with a deep brown-red color. It dissolves readily in cold chloroform and rather sparingly in cold benzene but readily on boiling. It is difficultly soluble in cold dry methyl alcohol, or dry acetone, somewhat more easily on boiling.

Subs., 0.1202: 13.2 cc. N (24.0°, 763 mm.).
Calc. for $C_{26}H_{30}O_3N_4$: N, 12.55. Found: 12.68.

4-Methoxyphenylazo-hydrocupreine.—The crude, washed dye was warmed with 85% alcohol, rapidly changing to the crystalline form. Recrystallized from amyl alcohol it forms deep red, glistening plates which decompose with gas evolution above 207°, sintering to a tar, and finally melting at 213–5° with further gas evolution. It dissolves more freely in chloroform than in the other usual solvents, and is readily soluble in hot benzene or amyl alcohol, sparingly in the cold. It yields a bright red solution in dilute acids and dissolves in conc. sulfuric acid with a deep purple color, changing to deep red, with a purple fluorescence.

Subs., 0.1409: 15.0 cc. N (21.0°, 754 mm.).
Calc. for $C_{26}H_{30}O_3N_4$: N, 12.55. Found: 12.27.

2-Ethoxyphenylazo-hydrocupreine.—The yield of the purified dye was equal to the hydrocupreine used. It forms a scarlet, amorphous powder, less easily soluble in ligroin than in the other usual neutral organic solvents. It softens at about 100°, melts gradually above 110°, and is completely fluid at 135–40°. It dissolves in dil. hydrochloric acid with a bright red color and is slightly soluble in cold *N* sodium hydroxide solution, more easily on boiling. The solution of the dye in conc. sulfuric acid is deep reddish purple by reflected light and purplish red by transmitted light.

Subs., 0.1146: 12.5 cc. N (31.0°, 758 mm.).
Calc. for $C_{27}H_{32}O_3N_4$: N, 12.17. Found: 12.22.

3-Ethoxyphenylazo-hydrocupreine.—The yield of the purified dye was slightly less than the amount of alkaloid used. It forms an orange-red powder which is less easily soluble in ligroin or ether than in the other usual neutral organic solvents. It is partly soluble in the cold in *N* sodium hydroxide solution and dissolves in dil. hydrochloric

acid with a deep orange-red color. It gives a deep, brown-red color in conc. sulfuric acid. The substance sinters and darkens above 75°, melts slowly above 90°, and is completely fluid at about 125°.

Subs., 0.1113: 11.7 cc. N (27.0°, 758 mm.).
Calc. for $C_{27}H_{32}O_3N_4$: N, 12.17. Found: 11.93.

4-Ethoxyphenylazo-hydrocupreine.—A ligroin solution of the purified, amorphous dye deposited the compound on standing as microscopic rhombs. However, the dye may be obtained as large, almost black rhombs by dissolving in hot 85% alcohol, cooling to 0°, diluting very cautiously with water until the initial turbidity just redissolves, seeding, and letting stand in the ice box. The crystals weathered in the air, probably owing to loss of water of crystallization. The pulverized crystals form a bright red powder, and, when anhydrous, darken and soften above 100° and melt at 156–61° when the bath is slowly heated. The substance melts when plunged into the bath at 130°. It dissolves in dil. hydrochloric acid with a bright red color, and is soluble with a purplish red color in hot, dil. sodium hydroxide solution. It dissolves quite easily in the usual neutral organic solvents with the exception of ligroin. The deep red solution in conc. sulfuric acid appears purple in thin layers and fluoresces purple.

Subs., 0.1369: 14.3 cc. N (21.5°, 755 mm.).
Calc. for $C_{27}H_{32}O_3N_4$: N, 12.17. Found: 12.03.

3,4-Methylenedioxy-phenylazo-hydrocupreine.—The dried, purified dye was dissolved in hot, dry acetone and the cooled solution filtered from a little insoluble matter and seeded with crystals obtained by allowing a test portion to stand in 70% alcohol. The substance separated on standing, the process being made more complete by the addition of small portions of water from time to time. As obtained in this way it forms deep maroon, apparently microcrystalline crusts which melt at 177–84° with slow gas evolution and slight preliminary darkening and softening. It dissolves in conc. sulfuric acid with an intense blue color and in dil. acids with a deep red shade. A suspension in boiling *N* sodium hydroxide solution disolves only incompletely, but on diluting an alcoholic solution with *N* sodium hydroxide a clear, deep red solution is obtained. It dissolves easily in cold

chloroform, less readily in the cold in dry methyl or ethyl alcohol, dry acetone, or benzene, but more freely on boiling.

Subs., 0.1255: 13.1 cc. N (24.5°, 763 mm.).
Calc. for $C_{26}H_{28}O_4N_4$: N, 12.17. Found: 12.02.

3,4-Dimethoxy-phenylazo-hydrocupreine.—Analysis showed that the usual purification process was insufficient. The dye was accordingly dissolved in acetone and treated with ligroin until about 10% had been precipitated as dark flocks. The mixture was filtered and the filtrate shaken out with water. The ligroin layer, from which most of the dye had separated, clinging to the walls of the separatory funnel, was shaken out with dil. hydrochloric acid and the solution filtered, diluted to large volume, and the dye precipitated with dil. ammonia. It forms a red, amorphous powder, which darkens above 90°, begins to melt at about 110°, and is completely fluid at about 180°. It dissolves in dil. acids with a bright red color, in dil. alkali with a cherry-red color, and is readily soluble in the usual neutral organic solvents with the exception of ligroin. The solution in conc. sulfuric acid is an intense red-violet in thick layers, and blue-violet in thin layers.

Subs., 0.1028: 10.8 cc. N (30.0°, 755 mm.).
Calc. for $C_{27}H_{32}O_4N_4$: N, 11.76. Found: 11.77.

3-Methoxy-4-ethoxyphenylazo-hydrocupreine.—Purification of the dye (from hydrocupreine and diazotized 3-methoxy-4-ethoxyaniline[1]) by the usual process failed to yield a pure product, so the dye was dissolved in a small volume of dry acetone and freed from a dark, flocculent impurity exactly as in the case of the dimethoxy compound. It forms a deep red powder which begins to darken and soften at about 80°, gradually melts above 100°, and is completely fluid at about 135°. It dissolves readily in the usual neutral organic solvents except ligroin, and is soluble in dil. sodium hydroxide with a cherry-red color and in dil. acids with a deep red color. The solution in conc. sulfuric acid is an intense violet, appearing blue in thin layers.

Subs., 0.1003: 9.8 cc. N (21.5°, 766 mm.).
Calc. for $C_{28}H_{34}O_4N_4$: N, 11.43. Found: 11.41.

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[1] This Journal, 41, 1461 (1919).

4 - Methoxy - 5 - ethoxyphenylazo - hydrocupreine. —4 - Methoxy - 5-ethoxyaniline[1] was diazotized, coupled with hydrocupreine, and the product purified exactly as in the case of its 3,4-isomer. The yield of dark red, amorphous product was 80% of the alkaloid taken. It was not entirely soluble in dil. sodium hydroxide solution and dissolved in dil. acids with a deep red color. It is less easily soluble in ligroin or ether than in the other usual neutral organic solvents. The dye dissolves in conc. sulfuric acid with an intense violet color, appearing redder by transmitted light than by reflected light. If softens and gradually melts above 75°, becoming completely fluid at about 120°.

Subs., 0.1308: 12.6 cc. N (23.5°, 769 mm.).
Calc. for $C_{28}H_{34}O_4N_4$: N, 11.43. Found: 11.23.

3,4-Dimethoxy-6-carboxyphenylazo-hydrocupreine.—After coupling 6-aminoveratric acid[2] with hydrocupreine in the usual way the deep red solution was treated with ammonium chloride, precipitating the dye (not the salt in this case) as a brick-red amorphous precipitate. This was purified with the aid of dil. hydrochloric acid and the filtrate treated with sodium acetate solution. The dye forms a deep maroon powder which, when rapidly heated to 235°, then slowly, melts and decomposes at 236°. It is rather difficultly soluble in the usual solvents and dissolves in dil. hydrochloric acid with a cherry-red color and in dil. sodium hydroxide with a somewhat brighter red shade. The color in conc. sulfuric acid is an intense violet.

[1] THIS JOURNAL, **41**, 1462 (1919).

[2] This substance, which does not seem to have been previously described, was prepared by nitrating methyl veratrate in acetic acid solution with fuming nitric acid, reducing to the amino ester (cf. Zincke and Francke, *Ann.*, **293**, 190 (1896)) with a solution of stannous chloride in methyl alcoholic hydrochloric acid, diluting with ice and water, and extracting the amino ester after making alkaline. Saponified with alcoholic sodium hydroxide on the water bath and evaporated, this yielded the acid as a mass of needles after diluting with water and acidifying with acetic acid. Recrystallized from ethyl acetate, it forms almost colorless, thick plates which dissolve with difficulty in boiling water and are somewhat more readily soluble in acetone than in the other neutral organic solvents. When rapidly heated to 185°, then slowly, it effervesces at 186°. An aqueous suspension gives an indigo-blue color with ferric chloride.

Subs., 0.1439: 9.2 cc. N (24.0°, 753 mm.).
Calc. for $C_9H_{11}O_4N$: N, 7.11. Found: 7.07.

Subs., 0.1224: 11.4 cc. N (27.0°, 757 mm.).
Calc. for $C_{28}H_{32}O_6N_4$: N, 10.77. Found: 10.56.

(*o-Phenylglycollic Acid*)-*p-azohydrocupreine*, *p*-$HO_2C.H_2C.O.C_6H_4N:NC_{19}H_{23}O_2N_2$.—The deep purplish red solution obtained on coupling *p*-aminophenoxyacetic acid[1] was acidified with a slight excess of acetic acid and the deep red precipitate filtered off and heated with 85% alcohol. The substance began to crystallize but dissolved entirely on adding sufficient boiling 85% alcohol. The solution was diluted with about $\frac{1}{2}$ volume of water, treated with a few drops of acetic acid, and seeded, and the product recrystallized from 50% alcohol, separating rapidly on seeding the cooled, supersaturated solution as brilliant, red needles containing 4 molecules of water of crystallization. An aqueous suspension dissolves only on adding a considerable excess of acetic acid, but dissolves easily on adding a drop or two of dil. sodium carbonate or hydroxide with a purplish red color, changing to orange-red on addition of hydrochloric acid. When rapidly heated to 195°, then slowly, the anhydrous acid darkens, softens, and sinters, finally melting at 199–200° with decomposition. It dissolves readily in chloroform or acetone, sparingly in dry methyl or ethyl alcohol, and gives a deep red color in conc. sulfuric acid, appearing purple in thin layers.

Subs., air-dry, 0.3940: loss, 0.0496 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{27}H_{30}O_5N_4.4H_2O$: H_2O, 12.82. Found: 12.58.
Subs., anhydrous, 0.1235: 12.6 cc. N (23.5°, 752 mm.).
Calc. for $C_{27}H_{30}O_5N_4$: N, 11.43. Found: 11.62.

m-Carboxamido-phenylazo-hydrocupreine, *m*-$H_2NOCC_6H_4N:NC_{19}H_{23}O_2N_2$.—The dye from *m*-aminobenzamide[2] was purified in the usual way, forming an amorphous powder which sinters and darkens above 135°, begins to melt at 160°, and is completely fluid and decomposes at about 195°. It dissolves readily in alcohol, less easily in dry chloroform or acetone or benzene, and dissolves in *N* sodium hydroxide solution with a deep cherry-red color.

Subs., 0.1077: 14.2 cc. N (24.0°, 757 mm.).
Calc. for $C_{26}H_{29}O_3N_5$: N, 15.25. Found: 15.09.

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[1] This Journal, **39**, 2196 (1917).
[2] *Ibid.*, **39**, 1438 (1917).

On rubbing the base with 1:1 hydrochloric acid it dissolves and the solution soon deposits the *dihydrochloride* as aggregates of flat, orange-red, microscopic needles.

p-Sulfophenylazo-hydrocupreine.—Sulfanilic acid coupled practically instantaneously with hydrocupreine under the usual conditions. Saturated ammonium chloride solution was added at the end in order to free the hydroxyl group. The scarlet *sodium salt* precipitated immediately, the amount increasing slightly on addition of sodium chloride. After washing with saturated sodium chloride solution a portion was recrystallized from water, separating on gradual cooling as glistening, scarlet rhombs and prisms which soon lose a portion of their water of crystallization on air-drying, turning a darker red and coming to equilibrium with 3.5 molecules of water of crystallization. The salt dissolves in water with a deep brown-red color, changing to cherry-red on adding alkali. Dilute aqueous solutions give slow-forming, red rhombs of the calcium and barium salts.

Subs., air-dry, 0.5788: loss, 0.0618 *in vacuo* at 80° over H_2SO_4.
Calc. for $C_{25}H_{27}O_5N_4SNa.3.5H_2O$: H_2O, 10.85. Found: 10.68.
Subs., anhydrous, 0.1213: 11.2 cc. N (24.5°, 764 mm.).
Calc. for $C_{25}H_{27}O_5N_4SNa$: N, 10.81. Found: 10.65.

A portion of the crude salt was dissolved in much hot water and acidified slightly with acetic acid, the *free sulfonic acid* soon separating as brilliant, brown-orange leaflets. Recrystallized from 50% alcohol it separates slowly as gleaming, red prisms containing 3 molecules of water of crystallization and dissolving with difficulty in boiling water. The anhydrous acid, when rapidly heated to 250°, then slowly, melts and decomposes at 252° with preliminary darkening and softening. It dissolves in sulfuric acid with a red-orange color, and is appreciably soluble in dry methyl alcohol, less readily in absolute alcohol or dry chloroform. An aqueous suspension dissolves readily in dil. mineral acids, but only on adding a large excess of acetic acid.

Subs., air-dry, 0.5158: loss, 0.0500 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{25}H_{28}O_5N_4S.3H_2O$: H_2O, 9.82. Found: 9.70.
Subs., anhydrous, 0.1293: 12.6 cc. N (26.0°, 762 mm.).
Calc. for $C_{25}H_{28}O_5N_4S$: N, 11.29. Found: 11.16.

m-Sulfonamido-phenylazo-hydrocupreine, *m*-$H_2NO_2SC_6H_4N{:}NC_{19}H_{23}O_2N_2$.—As obtained by the ordinary process of purification the dye from *m*-aminobenzene-sulfonamide[1] was still impure and it was accordingly dissolved in acetone and fractionally precipitated with ligroin, rejecting the first portions. The last fractions were dissolved in dil. hydrochloric acid and reprecipitated with ammonia, but the percentage of nitrogen was still low. The orange-brown, amorphous dye softens and sinters above 140°, gradually melts above 170°, and is completely fluid at about 190°. It dissolves in conc. sulfuric acid with an orange-red color, in dil. acids with an orange color, and in dil. sodium hydroxide with a deep cherry-red color.

Subs., 0.1018: 12.2 cc. N (30.5°, 757 mm.).
Calc. for $C_{25}H_{29}O_4N_5S$: N, 14.13. Found: 13.43.

p-Sulfonamido-phenylazo-hydrocupreine.—The dye obtained from *p*-aminobenzene-sulfonamide[2] was purified in the usual way. It forms an orange-brown amorphous powder which gradually melts above 155° and is completely fluid at about 190°. It dissolves in conc. sulfuric acid with a brown-orange color, in dil. acids with a red-orange color, and gives a deep cherry-red solution in dil. sodium hydroxide. It is readily soluble in alcohol, sparingly in benzene.

Subs., 0.1321: 15.7 cc. N (21.0°, 750 mm.).
Calc. for $C_{25}H_{29}O_4N_5S$: N, 14.13. Found: 13.62.

Quinolyl-6-azohydrocupreine.—The crude dye from 6-aminoquinoline crystallized suddenly when warmed with 85% alcohol. Recrystallized from amyl alcohol it separated slowly as aggregates of bright red, glistening, lenticular platelets with a golden reflex. When rapidly heated to 230°, then slowly, the substance melts and evolves gas at 232–4°, with preliminary softening and darkening. It dissolves readily in chloroform or boiling amyl alcohol, and is rather sparingly soluble in boiling alcohol, acetone, or benzene. An aqueous suspension does not dissolve on adding dil. sodium hydroxide, but an alcoholic solution, diluted with *N* sodium hydroxide, gives a clear, cherry-red solution. The solution in conc. sulfuric acid is reddish orange, while the color in dil. acids is orange-red.

[1] This Journal, **39**, 2428 (1917).
[2] *Ibid.*, **39**, 2429 (1917).

Subs., 0.1342: 17.1 cc. N (22.5°, 752 mm.).
Calc. for $C_{28}H_{29}O_2N_5$: N, 14.98. Found: 14.57.

(*B*) *Azo Dyes Derived from Hydrocupreidine.*

Phenylazo-hydrocupreidine.—13 g. of hydrocupreidine[1] were coupled with diazotized aniline in the usual way, completing the precipitation of the dye with ammonium chloride, and purifying by solution in very dilute hydrochloric acid and reprecipitation with ammonia. The yield was 13.9 g. When the amorphous product is dissolved in hot acetone it soon begins to crystallize and a portion for analysis was filtered from the hot solution, as the fractions deposited on cooling were accompanied by a little amorphous material. Recrystallized from alcohol it separates slowly on seeding as a hard crust of garnet-like rhombs which melt at 183–5° with slight preliminary darkening and softening. The substance forms a scarlet powder when crushed, and is appreciably soluble at room temperature in alcohol, methyl, ethyl, or amyl alcohol, or acetone, more easily on boiling. It dissolves readily in chloroform or benzene, and gives a reddish orange-brown color in conc. sulfuric acid.

Subs., 0.1110: 12.4 cc. N (22.5°, 772 mm.).
Calc. for $C_{25}H_{23}O_2N_4$: N, 13.46. Found: 13.11.

The Dihydrochloride.—When the amorphous, purified base is dissolved in not too little dry acetone and treated with an excess of conc. hydrochloric acid the dihydrochloride is obtained as an amorphous precipitate which crystallizes almost immediately. The salt was filtered off, moistened with 1:1 hydrochloric acid, and treated with dry acetone, filtering again and washing the orange-red platelets with dry acetone containing a drop of conc. hydrochloric acid. The air-dry salt contains 4 molecules of water of crystallization and is readily soluble in water. After drying *in vacuo* at room temperature over sulfuric acid and crushed alkali it turns a bright red, and, when rapidly heated to 215°, then slowly, decomposes at 220–1°, with slight preliminary darkening. It is readily soluble in dry methyl alcohol, less easily in cold chloroform, and dissolves with difficulty in cold absolute alcohol, more readily on warming.

[1] This Journal, **41**, 827 (1919).

Subs., air-dry, 0.6078: loss, 0.0788.
Calc. for $C_{25}H_{28}O_2N_4.2HCl.4H_2O$: H_2O, 12.84. Found: 12.97.
Subs., anhydrous, 0.1332: 13.2 cc. N (22.0°, 760 mm.). Subs., 01841: AgCl, 0.1074.
Calc. for $C_{25}H_{28}O_2N_4.2HCl$: N, 11.45; Cl, 14.49. Found: N, 11.48; Cl, 14.43.

α-Naphthylazo-hydrocupreidine.—The purified dye was recrystallized first from alcohol, then from a small volume of amyl alcohol, separating as tufts of deep red, hair-like needles which melt at 132–40° with preliminary darkening, softening, and sintering. It dissolves very easily in chloroform or benzene, somewhat less readily in dry acetone, and sparingly in cold methyl or ethyl alcohol, more easily on boiling. It dissolves in conc. sulfuric acid with a deep blue color, in dilute acids with a red shade, and gives a dull red color in dil. alkali.

Subs., 0.1220: 13.0 cc. N (23.5°, 761 mm.).
Calc. for $C_{29}H_{30}O_2N_4$: N, 12.02. Found: N, 12.28.

p-Nitrophenylazo-hydrocupreidine.—Hydrocupreidine and diazotized *p*-nitraniline were coupled exactly as in the case of hydrocupreine (see p. 343). The crude dye was purified in the usual manner and then crystallized readily when dissolved in boiling alcohol and allowed to cool.

Recrystallized from alcohol it separates on seeding, after cooling and filtering from a small amount of an amorphous impurity, as spherules of red microcrystals which melt to a tar at 135–9° and become completely fluid at 155–60°. It dissolves appreciably in cold methyl or ethyl alcohol, quite readily on boiling, and is very easily soluble in cold chloroform, acetone, or benzene. It dissolves in conc. sulfuric acid or dil. acids with a red-orange color, and in hot dil. *N* sodium hydroxide with a deep purplish red color. An alcoholic solution also gives a clear, deep purple solution when diluted with *N* sodium hydroxide.

Subs., 0.1192: 15.65 cc. N (27.5°, 764 mm.).
Calc. for $C_{25}H_{27}O_4N_5$: N, 15.19. Found: 15.00.

p-Sulfophenylazo-hydrocupreidine.—This substance was prepared in the same way as its hydrocupreine isomer. As the crude sodium salt

showed no tendency to crystallize it was dissolved in enough hot water to give a clear solution and acidified with acetic acid, seeding with crystals obtained by strongly acidifying a conc. test portion with acetic acid. Recrystallized first from water, then from 50% alcohol, the sulfonic acid separates slowly on seeding as minute orange-red crystals containing 5.5 molecules of water of crystallization, and dissolving appreciably in cold water, more easily on heating. The anhydrous substance is a darker red and, when rapidly heated to 245°, then slowly, it decomposes at 247°, with preliminary darkening and softening. It dissolves in conc. sulfuric or dil. acids with an orange color and in dil. sodium carbonate with a brown-red color, becoming more purple on adding sodium hydroxide. It is quite soluble in dry methyl or ethyl alcohol or chloroform and appreciably in cold, dry acetone.

Subs., air-dry, 0.3397: loss, 0.0533 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{25}H_{28}O_5N_4S.5.5H_2O$: H_2O, 16.64. Found: 16.28.
Subs., anhydrous, 0.1204: 11.7 cc. N (24.5°, 762 mm.).
Calc. for $C_{25}H_{28}O_5N_4S$: N, 11.29. Found: 11.18.

(*C*) *Derivative of 6-Hydroxyquinoline.*

Phenylazo-6-hydroxyquinoline.—Aniline was coupled as in the case of the phenolic alkaloids with an alkaline solution of 6-hydroxyquinoline. Precipitation of the dye was completed with ammonium chloride and the crude product recrystallized twice from alcohol, separating as minute, narrow, red leaflets which melt at 160–1.5° with slight preliminary softening, crystallizing again on cooling. In its solubilities, color reactions, and dyeing properties it resembles the corresponding hydrocupreine and hydrocupreidine compounds. The substance was first prepared by Mathëus,[1] who, however, gives no melting point.

Subs., 0.1297: 19.2 cc. N (28.5°, 762 mm.).
Calc. for $C_{15}H_{11}ON_3$: N, 16.87. Found: 16.81.

[1] *Ber.*, 21, 1642 (1888).

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CRYSTALLINE SALTS OF URIDINPHOSPHORIC ACID.

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(Received for publication, October 31, 1919.)

Uridinphosphoric acid was previously described in form of its crystalline brucine and barium salts.[1] The identification of a nucleotide in form of its brucine salts was found not sufficiently reliable. The crystallization of the barium salt of uridinphosphoric is a slow and tedious process. Hence it was desirable to find such salts of the nucleotide which could be crystallized with readiness when one is in possession even of only a small quantity of material. In this communication are described several such salts; namely, the mono- and the diammonium salts of uridinphosphoric acid, the neutral lead salt, and the brucine salt prepared from the crystalline ammonium salt.

The neutral ammonium salt is readily prepared from the brucine salt and crystallizes as heavy, elongated prisms. In aqueous solution its optical rotation is $[\alpha]_D^{20} = +21.0$. It crystallizes with one crystal water.

The mono-basic salt crystallizes in form of long, prismatic needles. It is very soluble in cold and hot water, and in hot glacial acetic acid. In aqueous solution it has an optical rotation of $[\alpha]_D^{20} = +13.0$. The air-dry substance contains no crystal water.

The neutral lead salt crystallizes in long needles, and was found very useful in obtaining pure uridinphosphoric acid when the original brucine salt had not been sufficiently purified.

The brucine salt was prepared in order to establish the constants of the compounds, since there was no conclusive evidence as to the absolute purity of the brucine salt obtained on fractionation of the mixed brucine nucleotides derived from the products of hydrolysis of nucleic acid.

[1] Levene, P. A., *Proc. Soc. Exp. Biol. and Med.*, 1917, xv, 21; *J. Biol. Chem.*, 1918, xxxiii, 229.

EXPERIMENTAL.

Di-basic ammonium salt was prepared by decomposing with aqueous ammonium hydroxide a solution of the brucine salt of uridinphosphoric acid in 35 per cent alcohol. The brucine salt of the mononucleotide was obtained either on acid or on ammonia hydrolysis of yeast nucleic acid.

The crude ammonium salt was dissolved in a minimum volume of hot water and to the solution hot methyl alcohol was added to very slight opalescence. On standing over night a deposit of heavy crystals formed. In subsequent experiments crystallization began immediately after addition of alcohol, if the solution was seeded with a crystal of the pure substance. The substance decomposed at 185°C. (uncorrected).

0.0994 gm. of the substance gave 0.1048 gm. of CO_2 and 0.0486 gm. of H_2O.

0.1000 gm. of the substance analyzed for Kjeldahl nitrogen estimation require 10.68 cc. of 0.1 N acid.

0.3000 gm. of the substance gave 0.0870 gm. of $Mg_2P_2O_7$.

	Calculated for $C_9H_{19}N_4PO_9 + \frac{1}{2} H_2O$.	Found.
	per cent	*per cent*
C	29.11	28.75
H	5.42	5.47
N	15.22	14.95
P	8.44	8.08

The optical rotation of the substance was as follows.

$$[\alpha]_D^{20} = \frac{+0.42 \times 100}{1 \times 2} = +21.0$$

Mono-basic ammonium salt was prepared in the following way: 2.0 gm. of the neutral salt were dissolved in 15.0 cc. of glacial acetic acid, and to the hot solution hot ethyl acetate was added dropwise. Care was taken to wait with further addition until the precipitate forming on contact of ethyl acetate with the solution had disappeared. After a slight opalescence was established, the solution was allowed to stand over night. A crystalline sediment was found in form of balls consisting of long needles. In subsequent experiments, if the solution in glacial acetic was seeded with the pure substance, crystallization began immediately. The air-dry substance contracted at 210°C. (corrected) and decomposed at 242°C. (corrected).

0.1000 gm. of the substance gave 0.1142 gm. of CO_2 and 0.0452 gm. of H_2O.

0.1000 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 8.8 cc. of 0.1 N acid.

0.3000 gm. of the substance gave 0.0943 gm. of $Mg_2P_2O_7$.

	Calculated for $C_9H_{16}N_3PO_9$.	Found.
	per cent	*per cent*
C	31.66	31.14
H	4.73	5.05
N	12.32	12.32
P	9.09	8.76

The optical rotation of the aqueous solution was as follows.

$$[\alpha]_D^{20} = \frac{+0.26 \times 100}{1 \times 2} = +13.0$$

Neutral lead salt was prepared in the following way: 2.0 gm. of the neutral ammonium salt were dissolved in 50 cc. of water; to the solution 10 cc. of glacial acetic acid were added, and to the hot solution of the nucleotide a hot solution of neutral lead acetate was added. Immediately a gelatinous precipitate formed which, on boiling, disappeared nearly completely. The solution was filtered and seeded with a few crystals obtained from a test-tube experiment. Crystallization in long needles began immediately. The crystals once formed are very little soluble in boiling water.

Dried to constant weight the substance had the following composition.

0.1070 gm. of the substance gave 0.0788 gm. of CO_2 and 0.0206 gm. of H_2O.

0.1856 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 6.90 cc. of 0.1 N acid.

0.2782 gm. of the substance gave 0.0573 gm. of $Mg_2P_2O_7$.

	Calculated for $C_9H_{11}N_2PO_9Pb$.	Found.
	per cent	*per cent*
C	20.40	20.08
H	2.10	2.21
N	5.29	5.21
P	5.86	5.74

Brucine Salt of Uridinphosphoric Acid.—About 4.0 gm. of the neutral ammonium salt were converted into the lead salt. This was freed from lead by means of hydrogen sulfide and the brucine salt was obtained in the usual way. The salt was recrystallized twice, each time out of 1,500 cc. of 35 per cent alcohol.

The air-dry substance effervesced without becoming transparent at 185°C. (corrected) and contracted and melted without further decomposition at 195°C. (corrected).

The air-dry substance had the following composition.

0.0996 gm. of the substance gave 0.1948 gm. of CO_2 and 0.0591 gm. of H_2O.

0.2000 gm. of the substance gave 12.8 cc. of nitrogen gas at $T° = 24°C.$, at $P = 759$ mm.

0.3000 gm. of the substance gave 0.0270 gm. of $Mg_2P_2O_7$.

	Calculated for $C_9H_{13}N_2PO_9(C_{21}H_{25}N_2O_4)_2+7H_2O$. *per cent*	Found. *per cent*
C	53.30	53.33
H	6.43	6.64
N	6.79	7.35
P	2.50	2.51

The rotation of the substance, owing to the insolubility of the substance, was taken in great dilution, and was as follows.

$$[\alpha]_D^{20} = \frac{-0.16 \times 100}{2 \times 0.4} = -20.0$$

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THE STRUCTURE OF YEAST NUCLEIC ACID.

IV. Ammonia Hydrolysis.

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(Received for publication, November 1, 1919.)

The tetranucleotide theory of the structure of yeast nucleic acid was first enunciated by the writer[1] and was subsequently conclusively demonstrated by the experimental evidence furnished by Levene and Jacobs,[2] and by Levene and La Forge.[3] The facts on which the theory was based were: first, the isolation of four nucleosides; second, the isolation of simple pyrimidine nucleotides. These were obtained on partial hydrolysis of yeast nucleic acid. The third fact, important for the development of the theory, was the elucidation of the order of linkage of the components of one simple mononucleotide.[4]

The experimental data obtained until that phase of work permitted no rational formulation of the mode of linkage between individual mononucleotides. Our original graphic representation of the entire molecule of yeast nucleic acid had only an arbitrary schematic sense. In a publication on thymus nucleic acid, Levene and Jacobs made that point clear. Owing to pressure of other work, our own investigations into the problem of the linkage of the mononucleotides was making slow progress, when Thannhauser with his collaborators,[5] and Jones with his collaborators[6] entered the field of nucleic acid study.

[1] Levene, P. A., *Biochem. Z.*, 1909, xvii, 121.

[2] Levene, P. A., and Jacobs, W. A., *Ber. chem. Ges.*, 1909, xlii, 2475, 2703; 1910, xliii, 3151; 1911, xliv, 1027.

[3] Levene, P. A., and La Forge, F. B., *Ber. chem. Ges.*, 1912, xlv, 608, 3164.

[4] Levene, P. A., and Jacobs, W. A., *Ber. chem. Ges.*, 1908, xli, 2704; 1909, xlii, 335, 1198.

[5] Thannhauser, S. J., *Z. physiol. Chem.*, 1914, xci, 329. Thannhauser, S. J., and Dorfmüller, G., *ibid.*, 1917, c, 121.

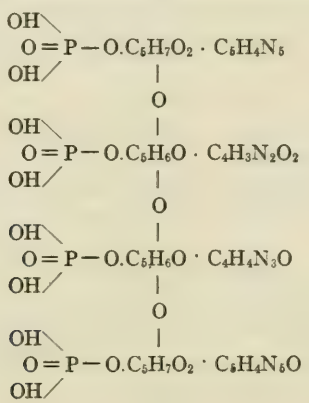
[6] Jones, W., and Richards, A. E., *J. Biol. Chem.*, 1914, xvii, 71. Jones, W., and Germann, H. C., *ibid.*, 1916, xxv, 93. Jones, W., and Read, B. E., *ibid.*, 1917, xxix, 123; xxxi, 39.

By means of either enzyme action or by methods of chemical hydrolysis, they obtained intermediate substances which were regarded by them as di- and trinucleotides.

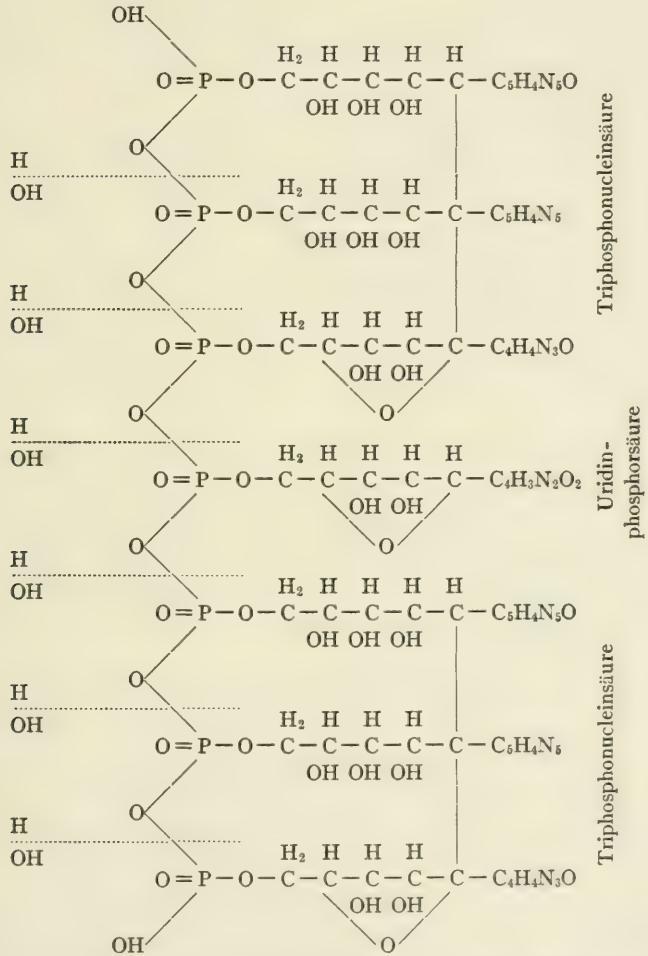
The methods of hydrolysis employed by them in some instances were identical with those employed by us in other instances, slightly modified. The method of separating simple nucleotides as their brucine salts was also introduced by us.

Thannhauser first announced the isolation of a trinucleotide from the products of digestion of nucleic acid by enzymes. Later, Thannhauser and Dorfmüller hydrolyzed nucleic acid by means of 25 per cent ammonia and supposedly cleaved the molecule into uridinphosphoric acid and a trinucleotide containing the remaining three nucleotides. On acid hydrolysis with 2 per cent sulfuric acid these authors obtained only uridinphosphoric acid. This publication appeared in Germany in 1917 and did not reach us until 1919. In 1914, Jones and Richards described experiments by which they thought they had cleaved nucleic acid into two dinucleotides; guanin-cytosine, and adenin-uridin dinucleotides. Subsequently, Jones and his coworkers described the same two dinucleotides which they obtained on hydrolysis of yeast nucleic acid by heating the acid in an autoclave in a 2.5 per cent ammoniacal solution for $1\frac{1}{2}$ hours at a temperature of 115°C. On the other hand, on hydrolysis by means of dilute acids, Jones and Read described a cytidin-uracil dinucleotide. On the basis of their respective findings, Jones and Thannhauser presented theories of the mode of linkage between the nucleotides.

According to Jones the structure of yeast nucleic acid is as follows:

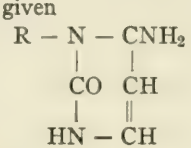


Thannhauser, on the other hand, presents the linkage of the nucleotides in the following way:[7]

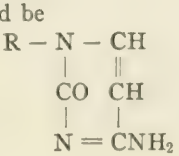


[7] In Thannhauser's representation there is an oversight in regard to the cytidin-linking.

It is given



It should be



These theories are based on the following considerations:

That of Jones on the assumption of the reality of the dinucleotides, and on the observations that the so called dinucleotides are tetrabasic. The theory of Thannhauser is based on the assumption of the belief in the reality of the trinucleotide, and second, on the fact that the so called trinucleotide is hexo-basic.

In previous communications we have criticized the conclusions of these writers, on the assumption that their observations were correct.[8] In a later communication,[9] we have shown that the cytidin-uridin dinucleotide was a mixture of uridin and cytidin mononucleotides. Uridinphosphoric acid was obtained as a crystalline barium salt. It may be mentioned here that optical rotation of the crystalline salt air-dry was $[\alpha]_D^{20} = 3.5$, or dry and barium-free $[\alpha]_D^{20} = 5.83$, whereas Thannhauser and Dorfmüller found for their uridinphosphoric acid + 14.4.

On the other hand the barium salt of the cytidinphosphoric acid had the optical rotation of $[\alpha]_D^{20} = +14.0$ or barium-free and dry $[\alpha]_D^{20} = +23.3$ which agrees with the recent finding of Thannhauser for the crystalline cytidinphosphoric acid, which was $[\alpha]_D^{20} = 23$. Thus it is possible that the substance described by Thannhauser as uridinphosphoric acid was of a lesser degree of purity than that of the cytidinphosphoric acid.

In a still later publication[10] we reported on the finding that the so called cytidin-uridin dinucleotide was fractionated by us into uridinphosphoric and adenosinphosphoric acids. The former was identified as the crystalline barium salt, the latter as the brucine salt, which at the time of that publication was converted into the barium salt. Since from a large quantity of brucine salt there was obtained only a small quantity of a barium salt analyzing satisfactorily for the salt of the adenosin nucleotide, the publication of the analytical data on that nucleotide was delayed It was subsequently found that adenosinphosphoric acid is identified most conveniently as the free acid. Crystalline adenosinphosphoric acid was described by Jones and Ken-

[8] Levene, P. A., *J. Biol. Chem.*, 1917, xxxi, 591.

[9] Levene, P. A., *Proc. Soc. Exp. Biol. and Med.*, 1917, xv, 21.

[10] Levene, P. A., *J. Biol. Chem.*, 1918, xxxiii, 425.

nedy;[11] the substance obtained by us differed from that of Jones only in the fact that our material dried in air contained no crystal water, whereas the substance of Jones contained one crystal water. The optical rotation of our substance is $[\alpha]_D^{20} = -38.5$. Whereas the rotation of adenosinphosphoric acid remains constant either in water or in 5 per cent ammonia water, the rotation of guanosinphosphoric acid shows a marked increase in its levorotation in ammonia water. This point may serve for differentiation between the two nucleotides. Thus it is proven that the adenin-uridin dinucleotide is a mixture of two mononucleotides.

The fraction which was originally regarded by Jones as a guanin-cytosine dinucleotide, and from which Read isolated an amorphous guanylic acid, was also found by us to consist principally of guanosinphosphoric acid. In addition a small proportion of uridinphosphoric acid was found in this fraction. The presence of the latter nucleotide might have escaped isolation if not for its recently described property of forming a crystalline lead salt.

The guanylic acid was isolated in a crystalline form, and constituted the greater part of the fraction.

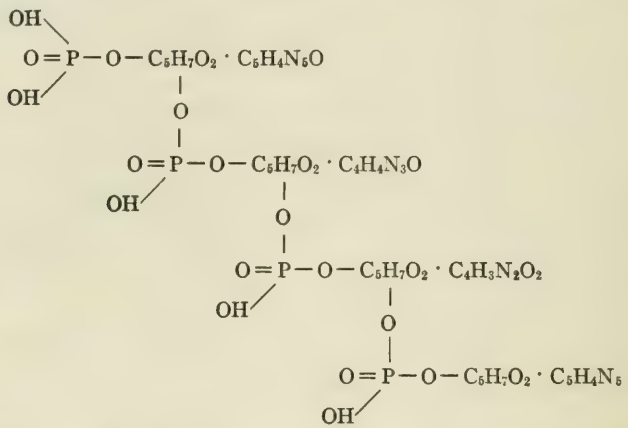
The method of hydrolysis employed by us consisted in heating the nucleic acid in a 2.5 per cent aqueous ammonia solution for 1 hour at 100°C. Thus the treatment was milder than the one employed by either Jones or Thannhauser. Thus the present findings nullify the experimental evidence in support of the theories of Jones and of Thannhauser. From the theoretical point of view, the theory of Thannhauser is not very tenable for the reason that a carbon to carbon-linking implies a very strong union, whereas the polynucleotide is readily dismembered into mononucleotides. Thannhauser, in fact, accepts it himself with great reserve. As regards the ether-linking accepted by Jones, it must be remarked that, as a rule, an ether-linking represents a very firm union. If one accepts that this rule does not apply to carbohydrates linked in ether form, he should present experimental evidence in support of this view.

However, if the work of Jones and of Thannhauser failed to support their speculations regarding the mode of linkage of the mononucleo-

[11] Jones, W., and Kennedy, R. P., *J. Pharmacol. and Exp. Therap.*, 1919, xiii, 45.

tides, it has been of great importance in furnishing further proof of the nucleotide structure of yeast nucleic acid; and also, in making it possible to show that the molecule of nucleic acid is readily decomposed into mononucleotides, and that the linkage between all nucleotides is of the same order.

On the basis of considerations such as these the linkage of the nucleotides could be expressed most simply in the following way:



For the present this form expresses the facts known about the structure of yeast nucleic acid. New facts and new evidence may cause its alteration, but there is no doubt as to the polynucleotide structure of the yeast nucleic acid.

It is unfortunate that, owing to war conditions, the work of Thannhauser was not known to us earlier, also that apparently our work was not known to Thannhauser.

EXPERIMENTAL.

The mode of hydrolysis was practically the same as that described in a previous communication,[10] with a difference in one detail; namely, the temperature of the autoclave was maintained at 100°C.

Treatment of the product of hydrolysis was also the same as described in that communication, and essentially the same as employed by Jones and his collaborators. The fraction precipitated by 98 per cent alcohol will be referred to as guanin fraction, and that remaining in solution as adenin fraction.

Adenin fraction was treated in exactly the same manner as that described in the previous communication. The brucine salt was recrystallized nine times with boiling 35 per cent alcohol.

The crystalline deposit consisted of uridinphosphoric acid previously described. The first three mother liquors, on concentration, gave a brucine salt containing C = 53.00, H = 6.40, and N = 10 per cent. The subsequent six mother liquors, on concentration, gave a brucine salt containing N = 8.5 per cent.

The brucine salt of the first three mother liquors was transferred into the ammonium salt. Originally the ammonium salts were converted into the barium salt. Barium salts, having analytical value sufficiently approaching that required by the theory, were obtained only after many purifications which were associated with much loss. Finally, an attempt was made to transform the ammonium salt into the free nucleotide. This was accomplished without difficulty in the following manner. To the hot solution of the ammonium salt, while the mixture was agitated, a hot solution of neutral lead acetate was added in a slow stream. When the necessary volume of lead acetate (25 per cent solution) was added, the mixture was brought to a boil and filtered. The precipitate was washed in a mortar and filtered; the operation was repeated three times. Finally the precipitate was suspended in water, treated with hydrogen sulfide, and the filtrate from lead sulfide was concentrated under diminished pressure at room temperature. On standing, adenosinphosphoric acid crystallized in long needles resembling the free nucleoside. The substance differed from that described by Jones in that it crystallized without crystal water. The analysis of the air-dry substance was as follows:

0.1010 gm. of the substance gave 0.1268 gm. of CO_2 and 0.0368 gm. of H_2O.

0.1000 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 14.26 cc. of 0.1 N acid.

0.3000 gm. of the substance gave 0.0940 gm. of $Mg_2P_2O_7$.

	Calculated for $C_{10}H_{14}N_5PO_7$. *per cent*	Found. *per cent*
C	34.57	34.24
H	4.07	4.08
N	20.17	19.96
P	8.94	8.73

The optical rotation of the substance in aqueous solution was

$$[\alpha]_D^{20} = \frac{-0.77 \times 100}{1 \times 2} = -38.5$$

In a solution of 5 per cent ammonia water the rotation was

$$[\alpha]_D^{20} = \frac{-0.80 \times 100}{1 \times 2} = -40.0$$

Hydrolysis of the Adenosinphosphoric Acid.—2 gm. of the substance in 35 cc. of 1 per cent sulfuric acid were boiled over flame with reflux condenser for 1 hour. The product of hydrolysis was neutralized with sodium hydroxide and to the neutral solution aqueous picric acid was added as long as a precipitate formed. The precipitate was dissolved in hot water and allowed to crystallize.

The analysis of the air-dry substance was as follows:

0.1000 gm. of the substance gave 26.2 cc. of nitrogen gas at $T° = 26°C.$ and $P = 752$ mm.

	Calculated for $C_5H_5N_5.C_6H_2(OH)(NO_2)_3+H_2O$. *per cent*	Found. *per cent*
N.....	29.32	29.60

The substance decomposed at 177°C. (uncorrected).

Brucine Salt of Adenosinphosphoric Acid.—2 gm. of the nucleotide were dissolved in hot water and the solution was neutralized with a solution of brucine in methyl alcohol. On coloring, the solution nearly solidified. The crystals of the brucine salt of the nucleotide were filtered off with suction, and the substance was recrystallized three times out of 35 per cent alcohol.

The air-dry substance on heating in a sealed capillary tube melted as follows: At 177°C. it began slightly to contract; at 195°, the substance effervesced, remaining perfectly colorless; at 225° a second point of effervescence was observed, the substance turning dark. The substance analyzed as follows:

0.1020 gm. of the substance gave 0.1978 gm. of CO_2 and 0.0572 gm. of H_2O.

0.2000 gm. of the substance gave 17.6 cc. of nitrogen gas at $T° = 24°C.$, $P = 769$ mm.

0.3000 gm. of the substance gave 0.0244 gm. of $Mg_2P_2O_7$.

	Calculated for $C_{10}H_{14}N_5PO_7.(C_{23}H_{26}O_4N_2)_2 \cdot 7H_2O$. *per cent*	Found. *per cent*
C	53.28	52.88
H	6.40	6.28
N	10.00	10.23
P	2.47	2.27

The rotation of the substance was

$$[\alpha]_D^{20} = \frac{-0.74 \times 100}{1 \times 2} = -37.0$$

The guanin fraction consisted principally of guanosinphosphoric acid (guanylic acid). It was treated in a general way in the manner indicated by Read. The lead salts were converted into the brucine salts, and these were fractionated in the same manner as the salts of the adenin fraction. However, the substance obtained from all the nine mother liquors contained over 10 per cent of nitrogen and only the fraction constituting the ultimate crystalline deposit contained on analysis about 8.75 per cent of nitrogen. Surprisingly also this fraction consisted in the main of guanylic acid. The brucine salts were converted into the ammonium salts. These were dissolved in boiling water and to the hot solution a hot solution of lead acetate was added in a slow stream. The mixture was then brought to a boil and filtered hot. The lead precipitate was freed from lead and concentrated under diminished pressure at room temperature. Generally an amorphous, somewhat gelatinous precipitate settles out. In some instances the solution turns into a semiliquid jelly. To bring about final crystallization, no general rule can be given. At times repeated precipitation with lead acetate will lead to a filtrate which, on concentration, solidifies into a crystalline mass. Often it is advisable to precipitate the nucleotide by means of lead acetate fractionally. The later fractions as a rule crystallize with less difficulty.

The properties and analysis of the crystalline guanylic acid were described in a previous communication.[12]

When the brucine salts with 8.75 per cent of nitrogen were converted into ammonium salts, and when these were taken up in hot water, part of the substance remained insoluble. This residue consisted of

[12] Levene, P. A., *J. Biol. Chem.*, 1919, xl, 171.

brucine salt which escaped, being converted into ammonium salt. The brucine salt on analysis showed a nitrogen content of N = 7.8 per cent. This brucine salt was then converted into the ammonium salt. The latter was dissolved in boiling water, and a hot solution of neutral lead acetate was added. The mixture was brought to a boil and filtered hot. The filtrate was seeded with a few crystals of the lead salt of uridinphosphoric acid, and allowed to stand near a hot water bath. It was found that when the cooling of the filtrate proceeded rapidly a gelatinous lead salt settled out. If, however, the cooling was progressing slowly the lead salt of uridinphosphoric acid settled out in crystalline form. For analysis the substance was dried to constant weight under diminished pressure at the temperature of xylene vapor. It analyzed as follows:

0.1118 gm. of the substance gave 0.0856 gm. of CO_2 and 0.0218 gm. of H_2O.

0.1848 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 7.7 cc. of 0.1 N acid.

0.2772 gm. of the substance gave 0.0574 gm. of $Mg_2P_2O_7$.

	Calculated for $C_9H_{11}N_2PO_9Pb$.	Found.
	per cent	*per cent*
C	20.40	20.73
H	2.10	2.18
N	5.29	5.84
P	5.86	5.78

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A STRAIN OF CONNECTIVE TISSUE SEVEN YEARS OLD.

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Plates 51 to 55.

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In July, 1914, Dr. Carrel reported the condition of a strain of connective tissue 28 months old, isolated from a fragment of heart extirpated from a chick embryo on January 17, 1912.[1] Today this strain is still alive. It has been under cultivation *in vitro* for a period of over 7 years and has undergone 1,390 passages.

The purpose of this article is to describe the technique employed in perpetuating the strain during the last 5 years and in measuring the increase of the tissue, the factors which influence the rate of growth, and the present condition of the strain.

Technique.

The technique does not differ fundamentally from the technique already reported.[2] The fragments of the old strain are allowed to grow undisturbed for 48 hours and are then divided into two parts and transferred to a fresh medium in the following manner.

The cover-glass is lifted with the heated point of a blunt cataract knife. It is then placed upon a piece of black glass. The fragment is extirpated by four clean cuts made with the blade of a sharp cataract knife within the area of new growth, and divided into two or three pieces as equal in size as possible. The pieces are transferred, with the point of the knife and a needle if necessary, to a bath of Ringer's solution. They are allowed to remain in the bath for about 45 seconds. Meanwhile the substances composing the medium are dropped upon a cover-glass and thoroughly mixed with the end of the cataract knife. Then the medium is spread over the surface of the glass, in

[1] Carrel, A., *J. Exp. Med.*, 1914, xx, 1.

[2] Ebeling, A. H., *J. Exp. Med.*, 1913, xvii, 273.

order that the depth and area of the clot may be approximately uniform in all preparations.

The fragments of tissue are transferred by means of the knife point to the medium. They must be embedded thoroughly in it, without folding or curling. This step must be carried out rapidly to guard against embedding after coagulation has set in; that is, after 15 to 20 seconds, under ordinary conditions of room temperature and moisture. Coagulation is allowed to proceed and occurs in from 45 to 50 seconds. During this period, as well as during the period of washing in Ringer's solution, the preparations are kept under a large Petri dish in order to eliminate as nearly as possible chance bacterial contamination from the dust of the atmosphere.

After coagulation the cover-glass is inverted and placed on a hollow slide and held in place by a small quantity of vaseline. When coagulation does not occur promptly, the slide is prepared with vaseline in the manner described above, but instead of inverting the cover-glass over the slide, the slide is inverted and placed over the cover-glass, care being exercised not to touch the periphery of the medium. The slide is then picked up with its adherent cover-slip and set aside until coagulation occurs. This procedure prevents undue evaporation, which occurs if the medium is exposed for any length of time to the surrounding air, even though kept under the large Petri dish. The slides are sealed with paraffin melting at 56°C. and placed in the incubator at an average temperature of 39°C.

The medium used for perpetuating the strain is composed of equal volumes of chicken plasma and chick embryo extract. This combination produces a clot firm but not dense enough to interfere with the migration of the cells. Although a mixture of one volume of extract and two volumes of plasma constitutes a satisfactory medium, it has been observed that the new growth is less extensive. The clot is firmer and contracts more closely around the tissue fragment. It seems that the central portion of the tissue is not reached by fresh medium. The cells composing it die, and the necrotic tissue appears to retard the growth of the peripheral portion of the culture.

The plasma is obtained from adult chickens. The blood is taken from the carotid artery through an oiled glass cannula, received in chilled paraffined tubes, and centrifuged. Finally, the supernatant

plasma is pipetted off and kept in tightly corked paraffined tubes in cold storage at 4°C. The best plasma is obtained from young, healthy adult chickens, not over 2 years old, which have not been fed for a period of 24 hours previous to bleeding. Blood withdrawn from more recently fed chickens gives a turbid plasma rich in fat globules. Such plasma gives a hazy clot in which the cells do not develop so well as in a clear plasma.

The tissue extract employed is obtained from 7 to 8 day chick embryos. The embryos are first washed in Ringer's solution to remove the amniotic fluid and traces of blood which adhere to them after removal from the shell. Then the solution is drawn off in order to obtain an undiluted tissue juice. The embryos are minced in a watch-glass with sharp, curved scissors. The pulp is centrifuged for 10 minutes and the supernatant fluid obtained is drawn off.

The area of new growth is measured by means of a projection apparatus. In adjusting the condenser it is essential to guard against the action of the light rays upon the tissues, since the heat developed by concentration of the radiations is injurious. Diffused light sufficiently powerful to give proper illumination without any noticeable ill effect must be used. The image of the fragment of tissue is cast upon a sheet of paper, and by means of a pencil the outline of the original fragment and that of the new tissue can be traced. Shortly after the cultures are prepared, a drawing of the outline of the piece of embedded tissue is made, which requires not more than 20 seconds. The culture can then be returned to the incubator. Subsequent tracings, after growth is well established, are made at intervals on the same sheet of paper. They must be made rapidly because, after a short time, small droplets form and settle in the concavity of the slide. These droplets, usually after a period of from $1\frac{1}{2}$ to 2 minutes, coalesce and then no longer interfere with focusing. Such a long exposure of the tissue to the light has probably a retarding effect on the growth and should be avoided.

The rate of growth is expressed in function of the initial area for a given time interval. That is, the area of the newly grown tissue is equal to n times the initial area, as, for instance, it may be said that it has doubled or tripled, or that it is 10 times larger, within the same interval of time. Then, the rates of growth of two fragments of tissue

of unequal size can easily be compared. For example, the area of a 7 sq. cm. fragment, and of a 5.8 sq. cm. fragment, became in 48 hours, respectively, 99 sq. cm., and 81.5 sq. cm. The first one increased 92 sq. cm., the second only 75.7 sq. cm. However, the relation of the size of the growth to the size of the initial area is identical:

$$\text{No. 1,}\quad \frac{99-7}{7} = 13.1 \qquad \text{No. 2,}\quad \frac{81.5-5.8}{5.8} = 13.1$$

Each fragment has become 13.1 times as large as it was. The relation existing between the rates of growth of two fragments can be expressed in the form of a ratio, which is equal to $\frac{13.1}{13.1} = 1$ in the above example.

Factors Which Influence the Rate of Growth.

The strain is used chiefly for measuring the influence of different factors on the rate of growth of connective tissue. The value of the method depends entirely on the property of the parts of a divided fragment of tissue to grow at the same rate when they are cultivated in identical media. Therefore it is important to know what factors may cause two pieces of the same fragment to grow at unequal rates, and to recognize the presence of these factors after the fragments have been embedded in their medium.

As a rule, when two parts of a fragment of the strain which has been growing actively for 48 hours are cultivated in identical media, they grow at the same rate. However, it was found that parts equal or unequal in size coming from the same original fragment do not always grow at the same rate. The reasons for this difference have been determined in such a manner that examination of the pieces makes it possible almost always to foresee whether they will grow at the same rate, and then to discard the useless cultures.

Fragments identical in area may differ in thickness. This may be seen easily by microscopic examination, and sometimes by the naked eye. When a region whiter than the surrounding tissue is seen, it is probable that it is thicker and possibly necrotic. Microscopic examination of the tissue then shows a spot very much darker in color. Tissues presenting these characteristics should be discarded. They

probably would not grow at the same rate as tissues of homogeneous appearance, identical in area.

The condition of the edges of the fragments is also an important factor of the rate of growth. If the incision has been made through the growing area of the tissue, the periphery of the fragments has everywhere a similar appearance. The incision can be seen easily with the microscope if it has been made in places through the plasma not yet invaded by the new cells. Very often the old plasma is slightly folded. As the rate of growth is slower under these conditions such cultures should be discarded.

The value of the growth of a piece of tissue is compared only with the value of the growth of another piece of the same tissue. Therefore the absolute value of the growth is not of great importance, and the factors which do not modify the relative value of the growth can be neglected. However, these factors exist and may increase or decrease in a large measure the extent of the growth. In measurements made on 142 cultures, it was found that the surface of a fragment may increase from 4 to 40 times the initial size. These large differences in the amount of growth are due to the quality of the plasma and of the embryo extract used for the medium, to the previous condition of the strain, to the temperature of the incubator, and to many other factors. They are of no great interest, since they act at the same time on both the fragments which are to be compared. From a practical point of view, the factors which may influence independently the rate of growth of the two halves of an original fragment are almost completely eliminated, if the cultures containing pieces of old plasma or fragments of tissue of unequal size and thickness are discarded.

Present Condition of the Strain of Connective Tissue.

After more than 7 years of life outside the organism, the rate of growth of the strain of connective tissue is very active. This rate seems to have increased progressively during the 7 years (Figs. 1 and 2), but it is possible that it may be only an apparent increase due to modifications of the technique. During the 1st year the growth was slow and irregular, because it was not yet known that the presence in the culture medium of certain substances contained in embry-

onic juices is essential for the permanent life of tissues *in vitro*. As soon as the tissues were washed in Ringer's solution, or in salt solution every 2 days, and embryonic juice was used in the plasma of the medium, the rate of growth increased very much and became almost constant.[2] When the strain was 28 months old, the fragments of tissue which showed the maximum speed in growth increased in 48 hours 15 times their area. The 7 year strain increases more rapidly, as the area of a fragment may become in 48 hours 40 times larger than that of the original fragment. But this difference does not prove absolutely that the amount of new tissue produced in a given time is greater than it was 5 years ago. The medium used today is composed of one volume of plasma and one volume of embryonic juice, while 5 years ago it was composed of two volumes of plasma and one volume of embryonic juice. Since the medium is less dense, the growing tissue is probably thinner, and the increase in area does not mean the production of more tissue than that produced several years ago. The morphology of the cells has not varied. The photographs made 2 years ago and a few months ago show about the same number of mitotic figures (Figs. 3 to 5).

During the last 5 years the rate of growth fluctuated under the influence of the composition of the medium and the condition of the tissues. The plasma varied according to the age and the condition of the chicken and the embryonic juices were not of constant quality. Many other factors retarded or accelerated the growth of the tissue during periods of varying lengths of time. But as soon as the strain was again cultivated in normal medium, it grew at the normal rate. As has been shown previously, since the activity of the cells is a function of the medium in which they are living, it is readily modified by altering the composition of the medium. When the rate of multiplication of cells decreased and the amount of new tissue became small, the strain could be brought to normal growth within a few weeks. It was always found that the decrease in the rate of growth was due to deficient medium, or extract, or to the presence of alkali at the surface of the slides.

CONCLUSION.

1. A strain of connective tissue is still very active after more than 7 years of life *in vitro*.

2. The rate of growth of the fragments of tissue can be measured accurately and used for testing the action of many different factors on the growth of connective tissue cells.

3. The rate of growth of the strain is at least as rapid as it was 5 years ago, and may be more active.

4. The connective tissue cells appear to have the power of multiplying indefinitely in a culture medium, as do microorganisms.

EXPLANATION OF PLATES.

PLATE 51.

FIG. 1. Culture 9211–1. Passage 1080. Stained December 31, 1917. 48 hours growth. × 16.

PLATE 52.

FIG. 2. Culture 13310–1. Passage 1367. Stained May 23, 1919. 48 hours growth. × 16.

PLATE 53.

FIG. 3. Culture 5835. Passage 732. Stained April 16, 1917. 24 hours growth. × 275.

PLATE 54.

FIG. 4. Culture 9211–1. Passage 1080. Stained December 31, 1917. 48 hours growth. × 275.

PLATE 55.

FIG. 5. Culture 12784–1. Passage 1347. Stained April 25, 1919. 48 hours growth. × 240.

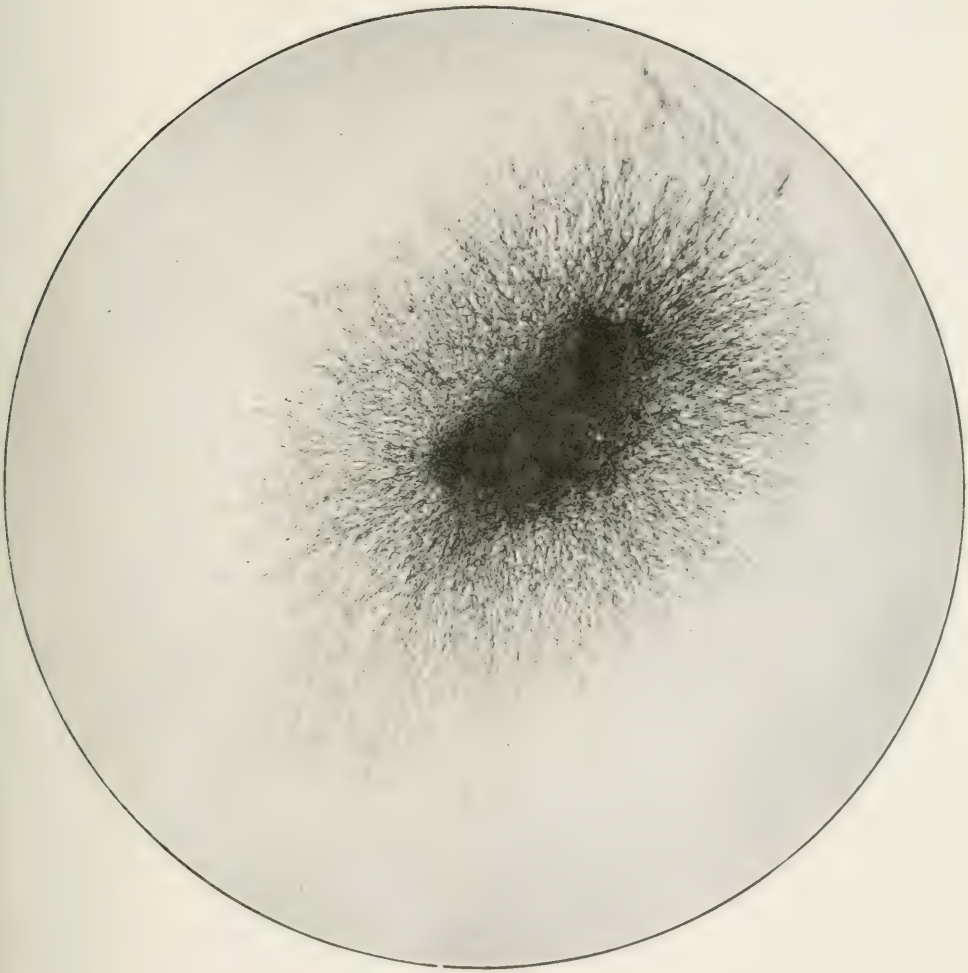

FIG. 1.

(Ebeling: Old strain of connective tissue.)

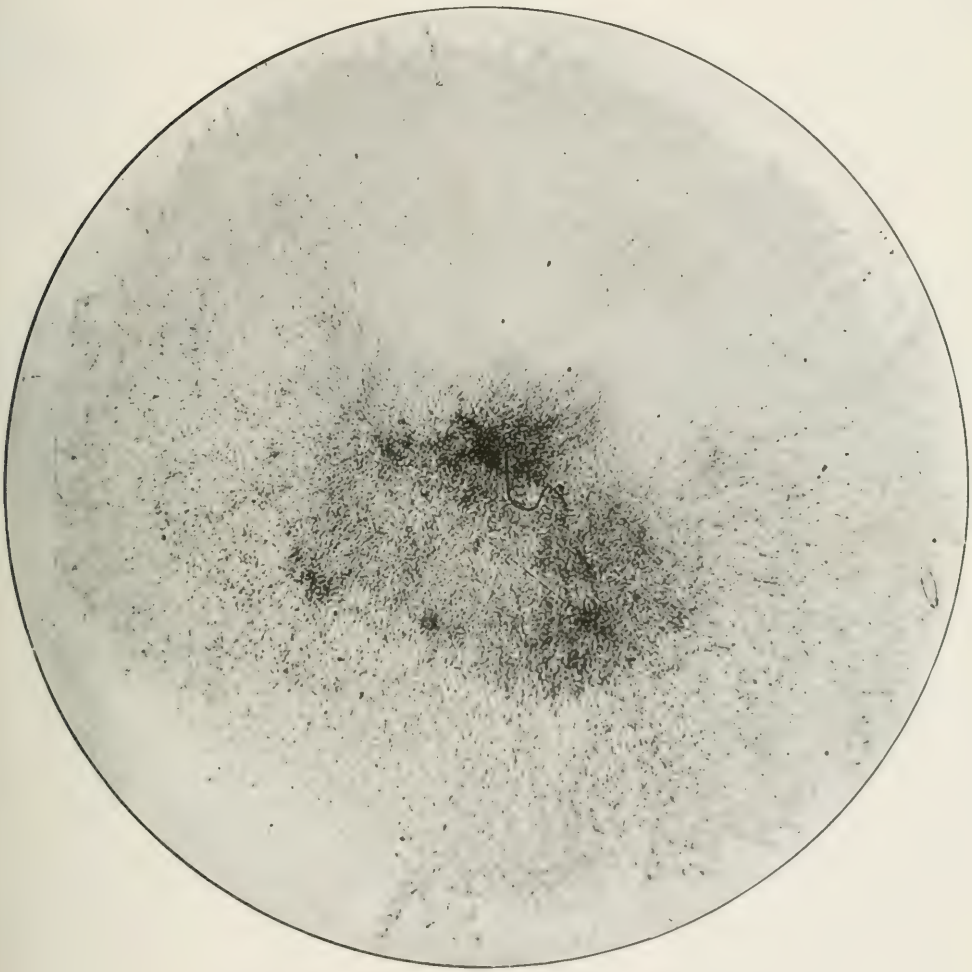

FIG. 2.

(Ebeling: Old strain of connective tissue.)

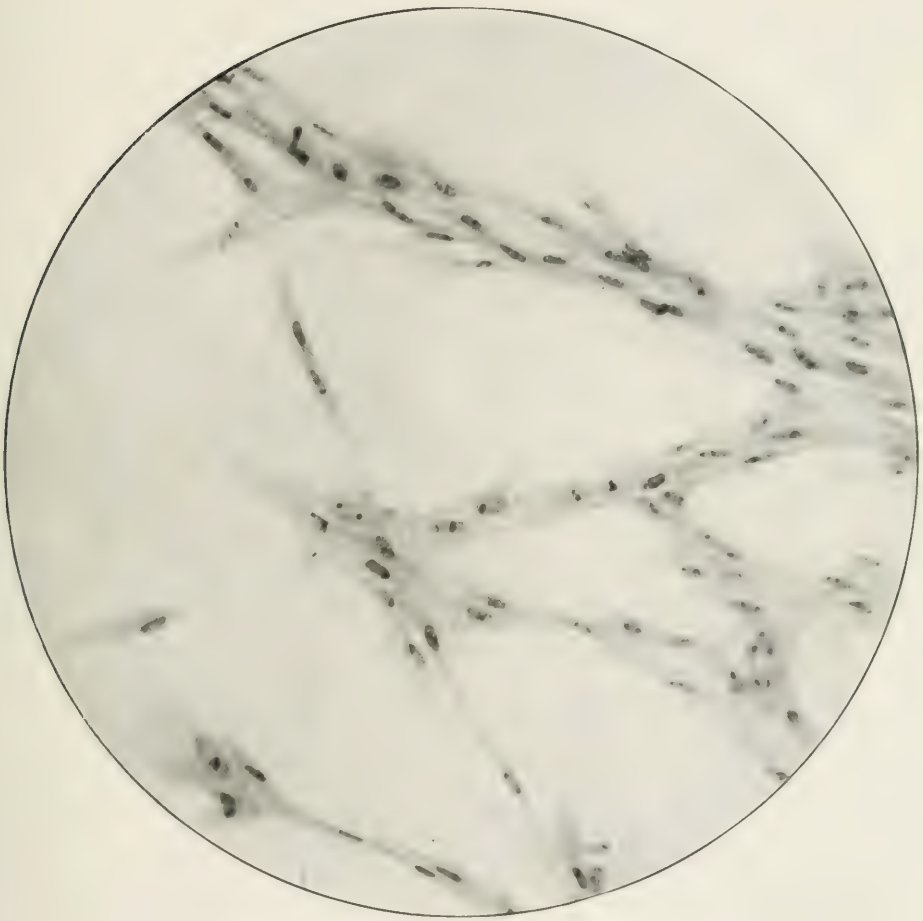

FIG. 3.

(Ebeling: Old strain of connective tissue.)

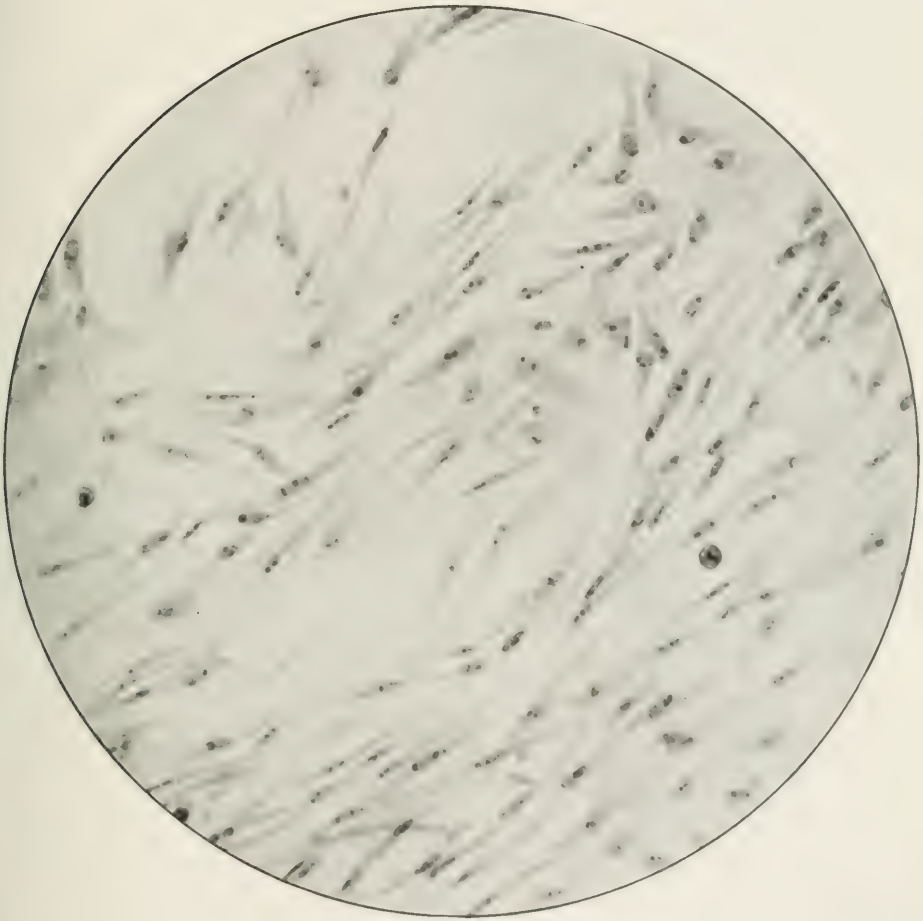

FIG. 4.

(Ebeling: Old strain of connective tissue.)

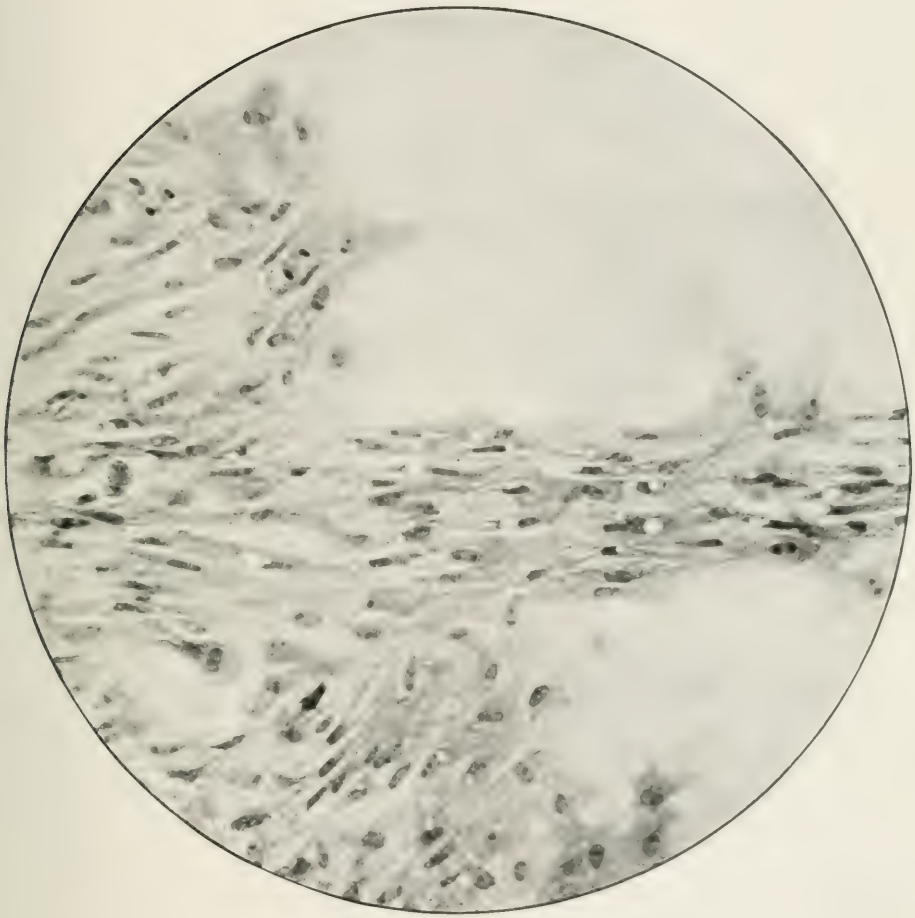

FIG. 5.

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ELECTRIFICATION OF WATER AND OSMOTIC PRESSURE.

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I. INTRODUCTION.

When a watery solution is separated from pure water by a strictly semipermeable membrane we call osmotic pressure the additional pressure upon the solution which is required to cause the migration of as many molecules of water from the side of the solution to the side of the pure solvent as migrate simultaneously from pure water to the solution. Van't Hoff's theory demands that this pressure should increase with the number of molecules in the solvent and that it should be equal to the gas pressure of the solute in the volume of the solution. The actual measurements of osmotic pressure of solutions of cane sugar made by Morse[1] and his collaborators as well as by the Earl of Berkeley[2] and his fellow workers show a rather close approximation to van't Hoff's theory.

When the solution is separated by a strictly semipermeable membrane, the difference in the rate of diffusion of water in opposite directions must be the greatest at the beginning of the experiment, and the difference must diminish steadily during the experiment as a consequence of the increase of hydrostatic pressure on the side of the solution. The value of the osmotic pressure of a solution as defined above must therefore vary with the difference in the rate of the diffusion of water molecules in the two opposite directions at the beginning of the experiment. When we double the concentration of the solute we also double the initial difference in the rate of diffusion of water molecules in opposite directions, and the additional pressure

[1] Morse, H. N., The osmotic pressure of aqueous solutions, *Carnegie Institution of Washington, Publication 198*, 1914.

[2] Earl of Berkeley, and Hartley, E. G. J., *Proc. Roy. Soc. London, Series A*, 1916, xcii, 477.

which is to be applied to the solution to make the rate of diffusion of water in both directions equal must also be doubled.

This influence of the concentration holds strictly only as long as the solute influences the rate of diffusion of water simply by the number of its molecules (*e.g.* in preventing a number of water molecules from impinging on the solution side of the membrane this number being equal to the number of molecules of solute impinging during the same time). Van't Hoff's law, however, must become inadequate if the molecules of the solute can modify the rate of the diffusion of water by other forces than mere gas pressure; *e.g.*, by electrical forces varying with the nature of the molecule. This is probably true for any solute but in a much smaller degree when the solute is a non-electrolyte than when it is an electrolyte; and in the case of electrolytes it holds in a smaller degree when the electrostatic field around the individual oppositely charged ions is nearly the same (*e.g.* in the case of NaCl) than when it is very different as in the case of $Na_4Fe(CN)_6$ or $LaCl_3$. This influence of the electrical field surrounding the ions upon the rate of diffusion is due to the electrification of the water molecules.

We use the term electrification of water merely as a short expression of the fact that electrostatic forces cause water to migrate in a definite sense through a membrane. Whether this electrification of water particles is due to a cluster formation of water molecules around an ion as a nucleus, or to some other cause, may for the present remain outside the discussion.

It is the purpose of this paper to show that the electrification of water molecules by ions in solution must in certain cases result in a deviation of the osmotic pressure of a solution from that to be expected on the basis of van't Hoff's theory; and that the sense and relative quantity of deviation can be predicted.

The writer has recently investigated the influence of various ions on the rate of diffusion of water through a collodion membrane separating pure water from a watery solution. Before being used for the experiment the collodion membranes were filled over night with a 1 per cent solution of gelatin and kept in water. The next day the gelatin solution was removed and the collodion flasks were rinsed a considerable number of times with warm water to remove

the remnants of the solution. The initial treatment of the membranes with gelatin apparently modified the collodion permanently since after weeks of daily use these membranes, treated once with gelatin, behaved differently from membranes not treated with gelatin. We shall return to this fact in another publication. An investigation of the influence of electrolytes on the rate of diffusion of water through such collodion membranes previously treated with gelatin has shown that all the phenomena observed can be explained on the basis of the following two rules.

"1. Solutions of neutral salts possessing a univalent or bivalent cation influence the rate of diffusion of water through a collodion membrane, as if the water particles were charged positively and were attracted by the anion and repelled by the cation of the electrolyte; the attractive and repulsive action increasing with the number of charges of the ion and diminishing inversely with a quantity which we will designate arbitrarily as the "radius" of the ion. The same rule applies to solutions of alkalies.

2. Solutions of neutral or acid salts possessing a trivalent or tetravalent cation influence the rate of diffusion of water through a collodion membrane as if the particles of water were charged negatively and were attracted by the cation and repelled by the anion of the electrolyte. Solutions of acids obey the same rule."[3]

These two rules allow us to predict in which sense the *nature* of the electrolyte in solution should modify the osmotic pressure of a solution calculated on the basis of van't Hoff's law. Suppose pure water is separated by a collodion membrane from a watery solution of an electrolyte. When the electrolyte is one of those mentioned in Rule 1, *i.e.* possessing a monovalent or bivalent cation, water is attracted by the solution and diffuses from the side of pure water into the solution, as if the particles of water were positively charged. They should therefore be attracted by the anion and repelled by the cation of the electrolyte and the more so the higher the valency of these ions. Hence water should diffuse more slowly into a solution of M/192 $CaCl_2$ than into a solution of M/128 NaCl, and more slowly into M/128 NaCl than into M/192 Na_2SO_4, and considerably more slowly into M/128 NaCl than into M/256 Na_3PO_4 or M/320 $Na_4Fe(CN)_6$. If, however, the water particles are negatively charged everything is reversed, the water diffusing more rapidly into M/192 $CaCl_2$ than

[3] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 720.

into M/128 NaCl, and more rapidly into M/128 NaCl than into M/192 Na_2SO_4.

From the theoretical connection between the relative rate of diffusion of water from pure water to the solution through a semipermeable membrane discussed before, it follows that the osmotic pressure of a solution should be modified by the *nature* of the ions it contains in the same sense as the initial rate of diffusion of water is modified. This idea can be put to a test by the choice of electrolytes for which the collodion membrane is strictly semipermeable; *e.g.*, gelatin salts. Gelatin solutions attain in bags of collodion an osmotic pressure which is permanent, provided the hydrogen ion concentration of the solution does not undergo any change during the experiment.

II. Analogies between Solutions of Gelatin and Aluminium Salts.

Gelatin is an amphoteric electrolyte which when the hydrogen ion concentration of its solution exceeds the critical value 2×10^{-5} N forms only salts of the type of gelatin chloride, gelatin sulfate, etc., while when its hydrogen ion concentration falls below this value it can form only salts of the form of metal gelatinates; *e.g.*, Na gelatinate, Ca gelatinate, and so on. At the critical hydrogen ion concentration 2×10^{-5} N—the isoelectric point for gelatin—it can exist only in the form of pure, *i.e.* non-ionogenic, gelatin.[4] In this condition gelatin is practically insoluble, practically non-ionized, and is practically incapable of producing any osmotic pressure. Both types of gelatin salts, metal gelatinates as well as gelatin chloride, etc., are very soluble, are strongly ionized, and are capable of producing osmotic pressure. The writer's experiments, which are not yet all published, have shown that for each given hydrogen ion concentration there exists a definite equilibrium between non-ionogenic gelatin, gelatin salt, and free acid. If we have 1 per cent solutions of isoelectric gelatin the ionized or salt portion of the gelatin is practically zero. When we add increasing quantities of an acid, *e.g.* HCl, an increasing portion of the gelatin is transformed into gelatin chloride, while the portion of non-ionogenic gelatin is correspondingly dimin-

[4] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 39, 237, 363, 483, 559.

ished. The relative proportion of gelatin salt and non-ionogenic gelatin depends therefore upon the hydrogen ion concentration of the solution and increases in a characteristic way with this concentration. This hydrogen ion concentration of the solution is, of course, not identical with the concentration of the acid added to the solution, since part of this acid is in combination with the gelatin forming the gelatin salt. Since practically only that fraction of the 1 per cent gelatin solution which is transformed into a gelatin salt produces an osmotic pressure it is obvious that the osmotic pressure of a 1 per cent solution of gelatin must change in a definite way with the hydrogen ion concentration of the solution. If we use different acids we find that different quantities of acid are required to bring a 1 per cent gelatin solution to the same pH.

When the hydrogen ion concentration is below the critical value 2×10^{-5} (or to use Sörensen's logarithmic symbol, when pH is > 4.7) a part of the non-ionogenic gelatin is transformed into metal gelatinate and this part is the greater the more the hydrogen ion concentration falls below 2×10^{-5}. There is again a definite equilibrium between hydrogen ion concentration, gelatin salt, and non-ionogenic gelatin.

Hence if we wish to compare the osmotic behavior of different gelatin salts we must see that the solutions have not only the same concentration of gelatin but also the same hydrogen ion concentration.[5]

The influence of solutions of gelatin on the electrification of water is the same as that of any other electrolyte and follows the two rules mentioned above. In solutions of metal gelatinates with monovalent or bivalent cations, water migrates through a collodion membrane as if the molecules of water were positively charged; in solutions of gelatin acid salts water migrates as if its particles were negatively charged. The turning point for the sense of migration seems to be near (or identical with) the isoelectric point of gelatin; *i.e.*, pH = 4.7.

[5] This latter point has been overlooked by the majority of colloid chemists who refuse to admit the chemical nature of the equilibrium between colloid and crystalloid, and who consequently ignore the rôle of the hydrogen ion concentration of the solution. Instead they compare the effect of the addition of equal quantities of different acids, overlooking the fact that they are thus comparing solutions in which the proportion of non-ionogenic gelatin and gelatin salt is different in the case of each acid.

It is perhaps not without interest that the behavior of gelatin is paralleled by the behavior of amphoteric electrolytes of a crystalloid character; *e.g.*, aluminium salts. Thus aluminium chloride exists only when the hydrogen ion concentration exceeds a certain critical value which seems to lie near that of the point of neutrality. When the solution becomes alkaline metal aluminates are formed. Na aluminate as well as $AlCl_3$ is very soluble. At the isoelectric point neither salt can exist and the insoluble $Al(OH)_3$ is formed.[6] The insoluble $Al(OH)_3$ has no osmotic pressure (or does not attract water) while solutions of both $AlCl_3$ as well as $NaAlO_2$ attract water powerfully. In the presence of $AlCl_3$ water molecules are apparently negatively electrified and in the presence of $NaAlO_2$ water shows positive electrification. The turning point for the sense of migration of water molecules seems to lie near or at the isoelectric point of aluminium; namely, pH about 7.0. It would be very important if we could measure the permanent osmotic pressure of aluminium salts in collodion bags, but this is impossible since aluminium salts (with the exception of the insoluble $Al(OH)_3$) diffuse through collodion membranes. It is, however, possible to determine the influence of different aluminium salts upon the rate of diffusion of water through a collodion membrane and it is found that this influence obeys the two rules.

We have mentioned this analogy between an amphoteric crystalloid, $AlCl_3$ and $NaAlO_2$ on the one hand, and an amphoteric colloid, gelatin chloride and sodium gelatinate, on the other to show that the fact of diffusibility or non-diffusibility through a collodion membrane does not force us to assume that the gelatin salts form no true solutions.

III. Osmotic Pressure of Different Metal Gelatinates.

Only the metal gelatinates with monovalent and bivalent cations need to be considered since the gelatin salts with trivalent cation seem to be insoluble. Different metal gelatinates were prepared

[6] The sparingly soluble $Al(OH)_3$ may be called a colloid since it does not diffuse through parchment paper. Sodium aluminate and $AlCl_3$ are crystalloids since they diffuse rapidly through such a membrane.

from gelatin rendered isoelectric (in the way described in former papers) by adding LiOH, NaOH, etc., to the gelatin. About 18 cc. of 0.01 N NaOH or $Ca(OH)_2$ must be contained in 100 cc. of a 1 per cent solution of isoelectric gelatin to obtain a metal gelatinate with a pH of 7.0; *i.e.*, with neutral reaction. We shall call a solution containing 1 gm. of isoelectric gelatin in 100 cc. a 1 per cent gelatin solution notwithstanding the fact that the gelatin is caused to combine with an acid or base.

We will first show that the gelatin salts with monovalent and bivalent cations influence the rate of diffusion of water in the same sense as is done by common crystalloid salts with monovalent and bivalent cations. Since in the presence of such metal gelatinate water diffuses through a collodion membrane as if its particles were positively charged (as ascertained by experiments with a constant current) we should expect that water should diffuse more rapidly into 1 per cent solution of metal gelatinate with monovalent cations, Li, Na, K, NH_4, than with bivalent cations, Mg, Ca, Sr, Ba, etc. This is indeed the case. We proceeded in the same way as in our previous experiments. Collodion bags,[7] in the shape of Erlenmeyer flasks with about 50 cc. contents, and all with the same surface, were prepared in as uniform a way as possible. The flasks were closed with a perforated rubber stopper through the opening of which a glass tube (with bore of about 2 mm. diameter) serving as a manometer was pushed. These collodion flasks were filled with water distilled in a tin still and having a pH of about 5.2. The collodion bag was put into a beaker filled with a 1 per cent solution of a metal gelatinate and the pressure head of the water in the manometer tube was at the beginning of the experiment about 120 mm. of H_2O. The fall of the level of the water in the glass tube was measured in definite intervals. In Figs. 1 and 2 the levels are plotted as ordinates over the time elapsed since the beginning of the experiment. The experi-

[7] The permeability of the collodion bags was tested before each experiment by filling them with M/4 cane sugar and measuring the rate at which water diffused into them. When the water rose in the glass tube with a bore of 2 mm. in diameter to a height of about 110 mm. in 20 minutes the membranes were considered serviceable.

ments were made at a constant temperature of 24°. The two figures show that water diffuses more rapidly into Li and K gelatinate than into Ba and Ca gelatinate. Na and NH_4 gelatinate behaved like Li and K gelatinate. The pH of the gelatin solutions was 8.2 in this experiment.

A second method of testing the influence of different metal gelatinates upon the rate of diffusion consisted in determining that concentration which a cane sugar solution must have in order to balance

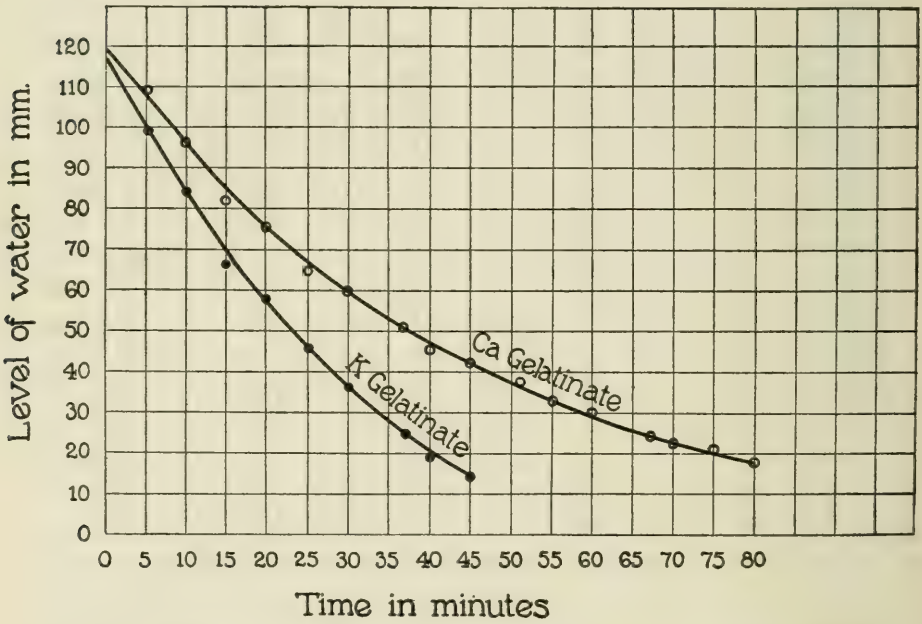

FIG. 1. Curves of fall of level of water when diffusing under an initial pressure head of about 120 mm. of H_2O against 1 per cent solutions of Ca and K gelatinate (pH = 8.2).

the attraction of a 1 per cent solution of metal gelatinate for water. The procedure was as follows. 350 cc. of a 1 per cent solution of a metal gelatinate, *e.g.* Na gelatinate, of pH 7.0 were put into each of a series of beakers and into each beaker was put one of the collodion flasks filled with a different concentration of cane sugar varying from

M/1 to M/64. It was useless to go below a concentration of M/64 of cane sugar since this was about the lowest concentration at which cane sugar influenced the rate of diffusion of water. The level of the cane sugar solution in the manometer tube was about 25 mm. at the beginning of the experiment. When the water diffused more rapidly from gelatin to cane sugar than in the opposite direction, the level in the manometer rose, when the water diffused more rapidly

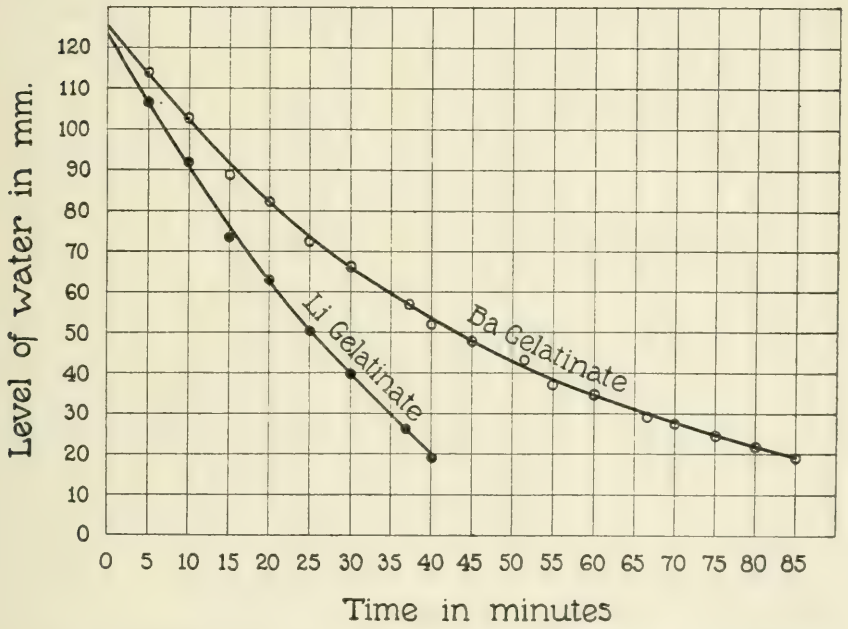

FIG. 2. Curves of fall of level of water when diffusing under an initial pressure head of about 120 mm. of H_2O against 1 per cent solution of Ba and Li gelatinate (pH = 8.2).

from cane sugar into gelatin than in the opposite direction the level in the manometer tube fell. Between the two was a concentration where the rate of diffusion in both directions was the same and this concentration of cane sugar we called the balancing concentration of cane sugar. Such experiments are only of value when of short duration on account of the fact that the sugar diffuses out into the gelatin

solution. In Table I the level of the water in the manometer tube after 30 minutes is given for the different 1 per cent gelatin solutions. The plus sign means a rise in the level of water in the cane sugar solution above the original level of 25 mm., the minus sign means a fall of the level of the sugar solution in the manometer tube.

We notice that the concentration which a cane sugar solution must have to balance osmotically 1 per cent solutions of gelatin salts with monovalent cations of a pH of about 7.0 lies between M/8 and 3M/32, while for Ca gelatinate of the same concentration and pH the balancing concentration of cane sugar has a value between M/32 and M/16,

TABLE I.

Nature of gelatin salt used.	Change of level of liquid in the manometer tube of different concentrations of cane sugar solution when immersed in 1 per cent solutions of different metal gelatinates of pH 7.0, after 30 min.										
	Concentration of cane sugar solution.										
	M/1	3M/4	M/2	3M/8	M/4	3M/16	M/8	3M/32	M/16	M/32	M/64
	mm.	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*
Li gelatinate	358	294	195	109	+60	+24	−4	−11	−16	−21	−16
Na "	397	289	251	120	+97	+22	−3	−11	−20	−19	−17
K "	379	285	186	102	+62	+22	−3	−7	−15	−18	−22
NH_4 "	390	323	222	127	+96	+35	+4	−4	−16	−19	−20
Ca "	397	300	241	172	+99	+69	+33	+16	+2	−2	−18
Ba "	376	310	225	145	+97	+77	+36	+29	+12	0	−15

and for Ba gelatinate a slightly lower value. We may, therefore, state that the balancing concentration of cane sugar is roughly over twice as great when the metal gelatinate has a monovalent cation as when it has a bivalent cation. Since water diffuses towards a cane sugar solution with a velocity which in the beginning of the experiment increases in proportion with the concentration of the sugar, we can say that the rate of diffusion of water into 1 per cent solutions of gelatin salts of the type Na gelatinate is between two and three times as great as the rate of diffusion of water into a solution of Ca or Ba gelatinate of the same concentration and pH.

The question now arises; Is there a similar difference between the

permanent osmotic pressure of 1 per cent solution of metal gelatinate when the cation is monovalent and bivalent? We have already published such measurements before we were aware of the fact that the electrification of water might play a rôle in osmotic pressure. In a paper published in this *Journal*[8] we have shown that when 1 per cent solutions of gelatin salts with monovalent cation—Li, Na, K, and NH_4 gelatinate—are separated by collodion membranes from distilled water they may reach a maximal osmotic pressure of from 300 to 325 mm. of gelatin solution, while the 1 per cent gelatin solutions with bivalent cation—Ca and Ba gelatinate—never reach an osmotic pressure higher than 130 mm.; *i.e.*, a little more than one-third the value of the highest osmotic pressure of 1 per cent solutions of gelatin salts with monovalent cation.

It seemed desirable to get the time curve for the rise in pressure, since such a curve permits a comparison between the influence of the cation on both the velocity of the diffusion of water from water into solution as well as on the pressure when osmotic equilibrium is reached. The experiment was as follows. The gelatin was rendered isoelectric in a way described in a previous paper. To 1 gm. of isoelectric gelatin were added 20 cc. of 0.01 N of an alkali (LiOH, NaOH, etc.) and then enough distilled water to bring the solution of the gelatin (which was melted) to 100 cc. The pH of these solutions in the experiment under discussion turned out to be about 8.8. The solutions were put into the collodion flasks and the latter were put into beakers containing 350 cc. of H_2O, the pH of which was raised at the beginning to about 9.0 by adding 0.2 cc. of 0.01 N NaOH to 350 cc. of H_2O, in order to prevent a rapid lowering of the pH of the gelatin solution through the CO_2 absorbed from the air by the distilled water of the outside solution. The temperature was 24°C. The pH decreased slowly in the beaker and as a consequence also in the gelatin solution, and in 20 hours the pH had fallen to about 7.0 in the gelatin solutions with monovalent cations, while it had fallen less in the gelatin solutions with bivalent cations. When the pH fell, the osmotic pressure also began to diminish on account of the shifting of

[8] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 483.

the equilibrium between non-ionogenic gelatin and the metal gelatinate formed.[9]

Fig. 3 gives the rise of level of liquid in the manometer during the first 12 hours for a 1 per cent NH_4 gelatinate and a 1 per cent Ba gelatinate solution. Equilibrium was reached after about 6 hours. The slight diminution of osmotic pressure due to the fall of pH on

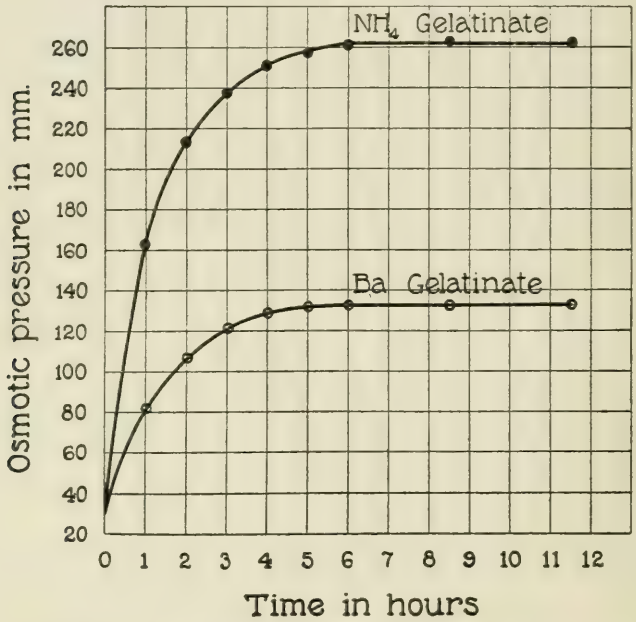

FIG. 3. Curves of rise of osmotic pressure of 1 per cent solutions of NH_4 and Ba gelatinate (pH = 8.8). Pressure in mm. of column of gelatin solution.

account of CO_2 action did not commence until later. The osmotic pressure of the 1 per cent solution of NH_4 gelatinate is in this experi-

[9] In order to make sure that the osmotic pressure reached is permanent, the experiments with metal gelatinates should be made with the exclusion of CO_2. Since, however, this source of error does not exist in the case of gelatin acid salts where the permanency of the final pressure reached can be made sure of, and since the results in that case are the same, as we shall presently see, and since the error was less for Ca and Ba gelatinate than for Na gelatinate, we need not dwell upon this point.

ment about twice as high as that of the 1 per cent solution of Ba gelatinate of about the same pH. During the first hour the relative rise of level in the solutions is also approximately as 1:2. Fig. 4 gives the curves for Na gelatinate and Ca gelatinate. The curve for Na gelatinate in Fig. 4 is identical with the curve for NH_4 gelatinate in Fig. 3 and the curve for Ca gelatinate in Fig. 4 is identical with the curve for Ba gelatinate in Fig. 3. The curves for Li and K gelatinate were identical with the curves for NH_4 and Na gelatinate in Figs. 3 and 4. Such results are always obtainable when both the

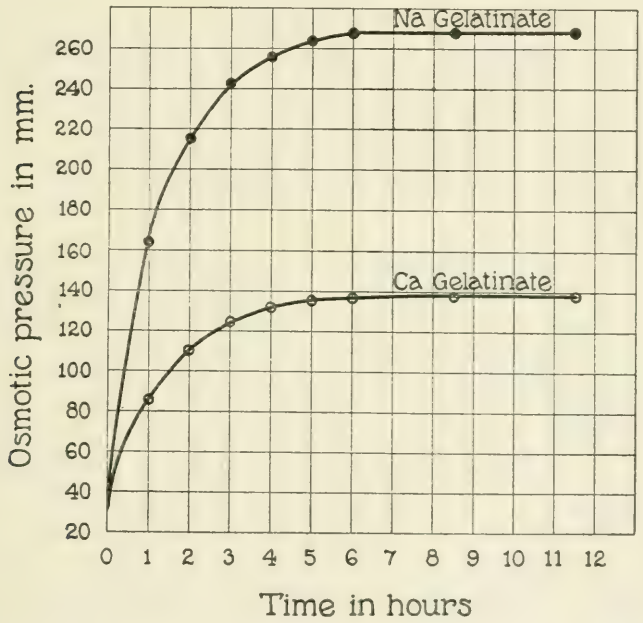

FIG. 4. Curves of osmotic pressure of 1 per cent solutions of Na and Ca gelatinate (pH = 8.8). Pressure in mm. of column of gelatin solution.

concentration and the pH of the solutions are identical. We therefore reach the conclusion that the different metal ions influence the osmotic pressure of gelatin solutions in the same sense as they influence the rate of migration of water into the solution. This supports the idea that the electrification of the water particles plays a rôle in the magnitude of the osmotic pressure as obtained by the use of semipermeable membranes.

IV. The Gelatin Cation.

When the hydrogen ion concentration of the gelatin solution is higher than 2×10^{-5} the gelatin can exist only in the form of gelatin chloride, sulfate, citrate, etc. Water is electrified negatively by such gelatin solutions and hence when pure water is separated by a collodion membrane from a 1 per cent solution of gelatin chloride or sulfate, etc., the water should diffuse more rapidly into the gelatin solution when the anion is monovalent than when it is bivalent. We have shown in a former paper[10] that all dibasic and tribasic acids (thus far tried by us), with the exception of sulfuric acid, combine in molecular and not equivalent proportions with gelatin. Thus gelatin forms with phosphoric acid a monogelatin phosphate, with tartaric acid a monogelatin tartrate; only with sulfuric acid does it form a digelatin sulfate. In the case of gelatin oxalate it is possible that we have a mixture of both the monogelatin and the digelatin oxalate, the former prevailing.

This fact is of great importance for our problem. In the case of monogelatin phosphate the anion is not trivalent PO_4, but essentially the monovalent anion H_2PO_4. The same is true for monogelatin citrate and in the case of monogelatin oxalate the anion is not a bivalent oxalate ion but a monovalent oxalate ion with one hydrogen attached. Only in the case of gelatin sulfate is the anion divalent. We have shown in our preceding paper that in the case of NaH_2PO_4 the attraction of the salt for water is of the order of magnitude of a salt with monovalent anion like NaCl, showing that the presence of the two H ions weakens the influence of the trivalent PO_4 ions considerably. We therefore should expect that in the case of salts like monogelatin citrate, monogelatin phosphate, monogelatin oxalate, and monogelatin tartrate the influence of anion on the rate of the diffusion of water should be of the same order of magnitude as in the case of gelatin chloride or nitrate; while in the case of digelatin sulfate the SO_4 ion should act as a divalent anion producing a stronger repelling effect on the negatively charged water than is done by gelatin chloride. This turns out as expected (Fig. 5).

[10] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 559.

When we fill the collodion bags as described with distilled water and put the bags into 1 per cent solutions of different gelatin acid salts of the same pH, giving the water inside the bag an initial pressure head of about 125 mm. of H_2O, the water will diffuse out more slowly against the gelatin sulfate than against any of the solutions of the other gelatin salts. This is demonstrated by Fig. 5. The abscissæ are the time in minutes, the ordinates the level of water in

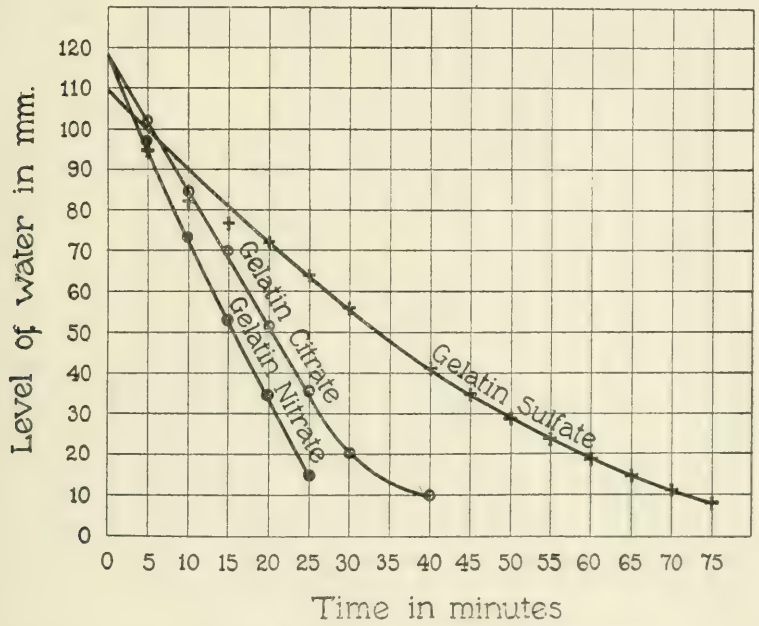

FIG. 5. Curves of fall of level of water when diffusing under an initial pressure head of about 120 mm. of H_2O against 1 per cent solutions of gelatin sulfate, gelatin citrate, and gelatin nitrate (pH = 3.5).

the manometer of the collodion flask. The initial pressure head was 125 mm. It required about 25 or 30 minutes for the pressure head of distilled water to fall from 120 mm. to about 20 mm. when it diffused into gelatin nitrate and monogelatin citrate, and 60 minutes when the distilled water diffused into gelatin sulfate.

The second method of testing the influence of the anion on the rate of diffusion of water consisted in determining the balancing

concentration of cane sugar for each of these salts in the way described. Table II gives the values observed after 30 minutes. The gelatin solutions had a pH of 3.5.

Using the same criterion as before we find that the balancing concentration of cane sugar lies for gelatin sulfate between 3M/32 and M/8 and for all the others between 3M/16 and M/4. In other words, the balancing concentration possesses for gelatin sulfate about one-half the value found for the other salts.

The writer has already published measurements of the osmotic pressure of 1 per cent solution of different gelatin salts. The osmotic

TABLE II.

Nature of gelatin salt used.	Change of level of liquid in the manometer tube of different concentrations of cane sugar solution when immersed in 1 per cent solutions of gelatin acid salts of pH 3.5, after 30 min.									
	Concentration of cane sugar solution.									
	M/1	3M/4	M/2	3M/8	M/4	3M/16	M/8	3M/32	M/16	M/32	M/64
	mm.	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*
Gelatin chloride	265	198	110	+15	+6	−18	−12	−22	−22	−19	−19
" nitrate	262	200	98	+25	+8	−11	−10	−19	−18	−19	−16
Monogelatin oxalate	273	203	146	+75	+33	0	−4	−12	−17	−15	−20
" tartrate	288	215	159	+47	+30	−4	−6	−20	−14	−16	−19
" phosphate	290	196	125	+45	+18	−8	−14	−19	−14	−13	−16
" citrate	335	244	115	+53	+30	0	−5	−9	−16	−24	−11
Digelatin sulfate	278	215	143	+74	+35	+10	+2	−8	−14	−14	−19

pressure varies for the same concentration of a gelatin salt with the pH of the solution and is a maximum at a pH of about 3.4. It was found that the maximum osmotic pressure of a 1 per cent solution of gelatin chloride, gelatin bromide, gelatin nitrate, gelatin acetate, monogelatin oxalate, monogelatin tartrate, monogelatin phosphate, and monogelatin citrate is about 320 mm. of the gelatin solution; while for a 1 per cent solution of gelatin sulfate the highest pressure obtainable is about 130 mm. This is in satisfactory agreement with the ratio we should expect on the basis of the influence of the anions on the rate of diffusion of the negatively electrified particles of water.

We considered it necessary to obtain also the curves for the increase

in the height of the level of water in the manometer with time until the equilibrium is reached. 1 per cent solutions of gelatin chloride, nitrate, oxalate, tartrate, citrate, and sulfate were prepared by making up the solution with the addition of as much of these acids to 1 gm. isoelectric gelatin as was required to produce a pH of 3.5. These 1 per cent gelatin solutions were put into collodion flasks containing the glass tube as described and each flask was put into a beaker with 350 cc. of distilled water the pH of which was made 3.0 by adding in each case the same acid as that of the gelatin. In Fig. 6 is plotted the rise and the final osmotic pressure of these solutions. The osmotic pressure reached in the case of gelatin sulfate was about 159 mm. while for the other salts it was almost twice as high (between 260 and 310 mm.). It is also noticeable from the curves that the relative velocity of rise during the 1st hour of the experiment also showed about the same ratio of almost 1:2 as the final equilibrium. The results thus confirm our expectation that on account of the electrification of the water molecules both the rate of diffusion as well as the final osmotic equilibrium are affected in the same sense.

If we wish to explain the differences between the observed osmotic pressure of solutions of calcium and sodium gelatinate or of gelatin chloride and gelatin sulfate, exclusively on the basis of van't Hoff's law, we are compelled to seek refuge in the assumption of the formation of aggregates of gelatin ions by which the number of these particles is diminished without diminution of the number of their charges.[11] This suggestion was first offered by Bayliss[12] when he found that the osmotic pressure of the colloid Congo red was considerably lower than was to be expected according to the molecular concentration and the conductivity of the solution. This assumption, however, which the writer had also tentatively accepted to explain the difference between the osmotic pressure of calcium and sodium gelatinate of the same concentration rests only on the facts which the assumption is supposed to explain, while the connection between rate of diffusion of water and osmotic pressure of a solution holds generally in all

[11] The writer has shown that the conductivity of 1 per cent Na gelatinate and of 1 per cent Ca gelatinate at the same pH is identical.

[12] Bayliss, W. M., *Proc. Roy. Soc. London, Series B*, 1911–12, lxxxiv, 229.

cases of dynamical equilibrium between two processes which occur simultaneously in opposite directions; *e.g.*, chemical equilibrium between reversible reactions.

Procter[13] and his collaborators have developed a theory of swelling of colloids based on the assumption that swelling is a purely osmotic phenomenon. On the basis of this theory we may consider the

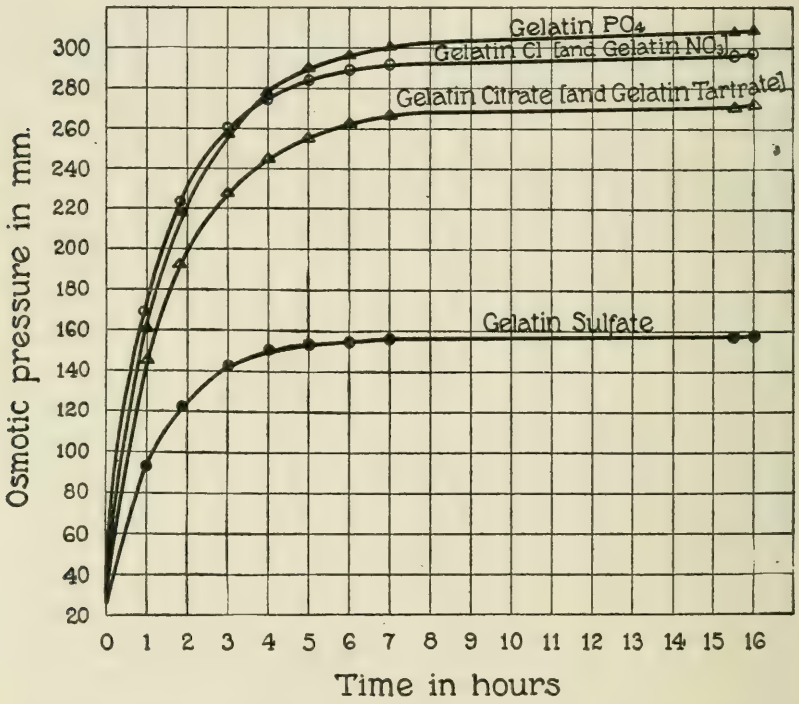

FIG. 6. Curves of osmotic pressure of 1 per cent solutions of gelatin phosphate, gelatin chloride (nitrate), gelatin citrate (tartrate), and gelatin sulfate (pH = 3.5). Pressure in mm. of column of gelatin solution.

surface and all the other solid parts of a block of gelatin to act as membranes permeable for water and crystalloids but not for gelatin. The osmotic conditions inside a solid block of gelatin which is

[13] Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307. Procter, H. R., and Burton, D., *J. Soc. Chem. Ind.*, 1916, xxxv, 404.

submersed in water are therefore comparable to those in our experiments except that we substitute a collodion membrane for the gelatin membrane.

The writer has shown that metal gelatinates of the same concentration of gelatin and of hydrogen ions swell much more when the metal in combination with the gelatin is monovalent (*e.g.* Li, Na, K, or NH_4) than when it is bivalent (*e.g.* Ca or Ba).[8] When gelatin is a cation the swelling is greater when the anion in combination with gelatin is monovalent (*e.g.* Cl, NO_3, or H_2PO_4) than when it is bivalent (*e.g.* SO_4).[10] The curves representing the influence of different acids and alkalies on the swelling of gelatin are similar to the curves representing the influence of the same acids and alkalies on the osmotic pressure of gelatin solutions. This similarity becomes clear if we adopt Procter's osmotic theory of swelling, adding to it our theory of the rôle of the electrification of particles of water in the phenomena of osmosis. In these experiments the solution surrounding the block of gelatin salt must have a low concentration of electrolyte since the presence of an excess of electrolyte suppresses the swelling, as pointed out in previous publications.

SUMMARY.

1. Amphoteric electrolytes form salts with both acids and alkalies. It is shown for two amphoteric electrolytes, $Al(OH)_3$ and gelatin, that in the presence of an acid salt water diffuses through a collodion membrane into a solution of these substances as if its particles were negatively charged, while water diffuses into solutions of these electrolytes, when they exist as monovalent or bivalent metal salts, as if the particles of water were positively charged. The turning point for the sign of the electrification of water seems to be near or to coincide with the isoelectric point of these two ampholytes which is a hydrogen ion concentration of about 2×10^{-5} N for gelatin and about 10^{-7} N for $Al(OH)_3$.

2. In conformity with the rules given in a preceding paper the apparently positively charged water diffuses with less rapidity through a collodion membrane into a solution of Ca and Ba gelatinate than into a solution of Li, Na, K, or NH_4 gelatinate of the same concentra-

tion of gelatin and of hydrogen ions. Apparently negatively charged water diffuses also with less rapidity through a collodion membrane into a solution of gelatin sulfate than into a solution of gelatin chloride or nitrate of the same concentration of gelatin and of hydrogen ions.

3. If we define osmotic pressure as that additional pressure upon the solution required to cause as many molecules of water to diffuse from solution to the pure water as diffuse simultaneously in the opposite direction through the membrane, it follows that the osmotic pressure cannot depend only on the concentration of the solute but must depend also on the electrostatic effects of the ions present and that the influence of ions on the osmotic pressure must be the same as that on the initial velocity of diffusion. This assumption was put to a test in experiments with gelatin salts for which a collodion membrane is strictly semipermeable and the tests confirmed the expectation.

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THE COMBINATION OF ENZYME AND SUBSTRATE.

I. A Method for the Quantitative Determination of Pepsin.

II. The Effect of the Hydrogen Ion Concentration.

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(Received for publication, July 7, 1919.)

I. A Method for the Quantitative Determination of Pepsin.

Considerable experimental evidence has been obtained by various authors to show that enzymes are removed from their solutions by insoluble substances.[1,2] Pepsin has been especially studied from this view-point. Dauwe[3] showed that this property of removing pepsin from its solution was not common to all substances and was connected in some way with the digestive action of the enzyme. He was also able to show that the size of the particles, at least in the case of egg albumin, was without any marked effect on the amount of pepsin removed.

Abderhalden[4] and his coworkers state that pepsin is removed almost completely from its solution by insoluble proteins on which it acts and they consider that this plays an important rôle in the kinetics of the reaction. These results were partially confirmed by Leary and Shieb.[5]

[1] For the literature see Bayliss, W. M., The nature of enzyme action, London, 3rd edition, 1914.

[2] Nelson, J. M., and Griffin, E. G., *J. Am. chem. Soc.*, 1916, xxxviii, 1111.

[3] Dauwe, F., *Beitr. chem. Physiol. u. Path.*, 1905, vi, 426. This paper reviews the earlier work.

[4] Abderhalden, E., and Steinbeck, E., *Z. physiol. Chem.*, 1910, lxviii, 293. Abderhalden, E., and Strauch, F. W., *ibid.*, 1911, lxxi, 315. Abderhalden, E., and Wachsmuth, F., *ibid.*, 339. Abderhalden, E., and Friedel, F., *ibid.*, 449. Abderhalden. E., and Kramm, F., *ibid.*, 1912, lxxvii, 462.

[5] Leary, J. T., and Shieb, S. H., *J. Biol. Chem.*, 1916–17, xxviii, 393.

Bayliss[1] attaches considerable importance to the combination of enzyme and substrate and considers it an essential point in the theory of enzyme action. Van Slyke and Cullen[6] were able to formulate the kinetics of enzyme action on the basis of the law of mass action by assuming the existence of a compound between enzyme and substrate.

The study of this combination, however, has not furnished any quantitative experimental data as to the nature of the reaction or the influence of various factors on it—due largely to the difficulty of determining quantitatively the amount of enzyme.

It seemed important, therefore, to obtain quantitative experimental data on this subject. In order to do this it was necessary to have a convenient and accurate method for the determination of pepsin. It was found that the change in the conductivity during the digestion of egg albumin by pepsin afforded such a method. Sjöqvist[7] found that there were marked changes in the conductivity during pepsin digestion. His results were confirmed with the exception that the change was found not to follow the actual rate of digestion. It can be used therefore only as an empirical method for the determination of pepsin and not for the study of the kinetics of the reaction, as was done by Sjöqvist. The reason for this divergence is probably due to the fact that the change in conductivity is due to two causes; (1) the liberation of free acid (carboxyl) groups which increase the conductivity, and (2) the liberation of free amino groups which bind some of the free acid and so decrease the conductivity. This explanation is borne out by the following facts.

With dilute solutions of egg albumin containing strong acetic acid (pH 2.3) there is a regular small increase in the conductivity which nearly parallels the increase in free amino groups as followed by the increase in amino nitrogen. This is due to the fact that, owing to the very large excess of free undissociated acid present, the ions which are removed from solution by combination with the free amino groups are replaced by the dissociation of more acid, and so kept nearly constant. The slight increase in conductivity is therefore due to the liberation of free carboxyl groups. In strong acid solu-

[6] Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1914, xix, 141.

[7] Sjöqvist, J., *Skandin. Arch. Physiol.*, 1893–95, v, 277.

tions, however, as hydrochloric, there is a rapid decrease in the conductivity—due to the removal of acid ions by combination with the liberated amino groups. This change is so much larger than the increase due to the acid groups set free by the protein that the increase in conductivity due to the latter is more than compensated. It has already been shown[8] that the actual rate of digestion is approximately the same in all acids at the same reaction so that the differences in the changes in the conductivity cannot be ascribed to differences in the rate of digestion.

It was found that the maximum change occurs in strong solutions of egg albumin titrated to pH 2.6 with hydrochloric acid. This solution was therefore used. The conductivity was followed by means of the apparatus described by Taylor and Acree.[9] The electrodes were of the dipping type and were immersed in the solution in order to make a reading. It was found that the percentage change in conductivity was constant for a given quantity of pepsin, irrespective of the absolute value of the original conductivity. The readings and figures are therefore given in terms of the increase in the scale readings on the bridge, which for small readings are equivalent to the percentage change. The measurements were carried out as follows.

25 cc. of a 3 per cent egg albumin solution were pipetted into a series of large "Non-sol" test-tubes and suspended in a water bath at 37° ± 0.02°. The electrodes were immersed in the solution and 1 cc. of the pepsin solution was added. The external resistance was then set so as to give a bridge reading of 500; *i.e.*, the middle of the bridge. The change in conductivity was now followed by the bridge readings. These figures are related to the actual change in resistance of the solution by means of the formula $\frac{X}{R} = \frac{A}{1{,}000 - A}$ where X = resistance of the solution, R is the external resistance, and A is the bridge reading.

The figures given in Table I are the increase in the value of A and are very nearly proportional to the percentage increase in the resistance.

[8] Northrop, J. H., *J. Gen. Physiol.*, 1918–19, i, 607.

[9] Taylor, W. A., and Acree, S. F., *J. Am. Chem. Soc.*, 1916, xxxviii, 2396.

Readings were taken at intervals so as to give points corresponding to changes of 2 to 4 units of the bridge reading (which could be easily read to 0.25 units). These points were then plotted on a large scale by means of a flexible "spline" and weights, so that the curves were

TABLE I.

Change in Conductivity of Solution of Egg Albumin with Varying Amounts of Pepsin.

25 cc. of egg albumin solution, pH 2.6. R 320.
Temperature 37° ± 0.02°C. A at beginning 500.

Relative amount of pepsin added in volumes of 1 cc.

100		50		25		12.5	
Time.	Increase in scale reading.	Time.	Increase in scale reading.	Time.	Increase in scale reading.	Time.	Increase in scale reading.
min.		*min.*		*min.*		*min.*	
3	4.0	4	3.0	3	1.5	7	2.2
8	8.5	13	7.8	17	6.0	9	2.7
14	12.0	23	11.0	28	8.0	26	5.2
18	14.5	39	16.0	46	11.5	34	6.7
31	21.0	51	19.5	60	13.5	67	9.2
43	26.0	88	26.5	93	18.5	102	13.2
83	38.0						

Change.		Time necessary to cause change.		
Increase in scale reading.	Time.			
	min.	*min.*	*min.*	*min.*
5 to 10	6.0	11.8	22.4	44.0
10 to 15	8.1	14.4	32.0	

Interval.	Relative amount of pepsin calculated.			
5 to 10	(100)	51	27	13.6
10 to 15	(100)	56	25	
Average.....	(100)	53	26	13.6

30 to 50 cm. long. The time necessary to cause a given change was then interpolated from these curves. In this way errors in individual readings were smoothed out and, as the curves are regular, accurate values for the time necessary to cause a given change could be ob-

tained. Since there is always some uncertainty about the zero reading the interval from 0 to 5 was omitted and the time to cause a change of from 5 to 10 and from 10 to 15 units was taken. The values obtained in this way were then averaged and the result taken as proportional to the reciprocal of the amount of pepsin present. By comparing this figure with the corresponding one from a control containing a standard amount of pepsin, the relative quantity of pepsin present could be determined, since it was found that the time

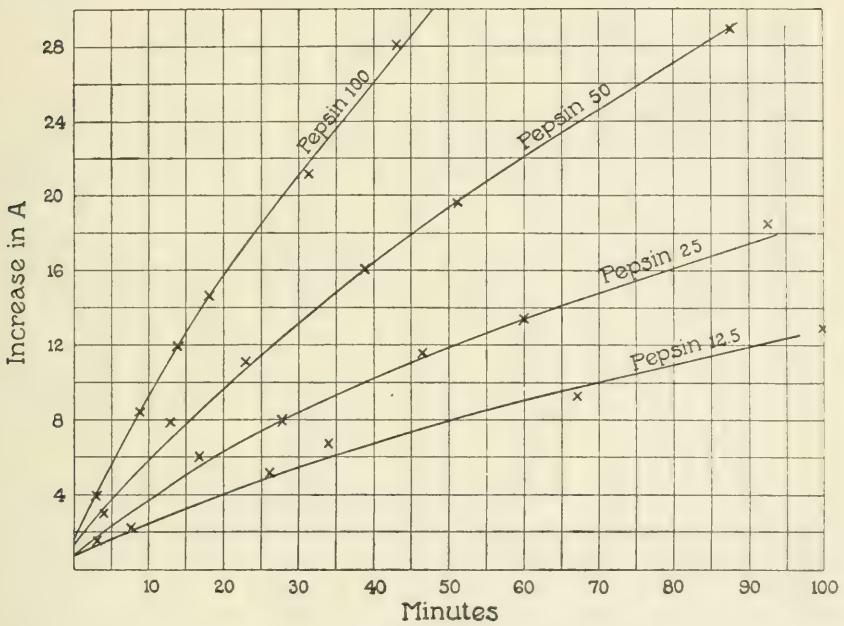

FIG. 1.

necessary to cause any given change was inversely proportional to the amount of pepsin present. That is, double the quantity of pepsin requires half the time, etc. (Arrhenius' "QT" rule).[10]

Table I and Fig. 1 give the results with a series of solutions containing different amounts of pepsin. It will be seen that the amount of pepsin can be determined with an accuracy of ± 2 to 3 per cent,

[10] Arrenhius, S., Quantitative laws in biological chemistry, London, 1915.

and further that ten to fifteen determinations can be made in the course of 1 to 2 hours depending on the amount of pepsin present.

It was now possible to determine quantitatively the amount of pepsin removed from solution by various substances.

Table II shows the results of such a series with coagulated and dried egg albumin. The control experiments show that the decrease in the amount of pepsin is not due to the destruction of the pepsin on standing or to the retarding effect of the products of reaction.

TABLE II.

Change in Concentration of Pepsin in Various Solutions.

Temperature 25°C.
pH of all solutions, 2.5.
1 cc. of solution pipetted off and pepsin estimated at time noted.

Time.	Relative amount of pepsin per cc.		
	20 cc. of pepsin solution.	20 cc. of pepsin solution + 1 gm. of egg albumin in solution.	20 cc. of pepsin solution + 1 gm. of coagulated egg albumin.
min.			
1	(100)	101	95
10	101	101	84
20	94	96	82
40	95	100	86
80	100	128*	80
160	97	98	86

* This is an experimental error. The curve was irregular and gave widely divergent results for different intervals.

A series of experiments was now made with various substances. The results are summarized in Table III. It is obvious that the removal of the pepsin is not purely a matter of surface but that it is dependent in some way on the substance itself.[11]

This fact is brought out more strongly in the experiments summarized in Table IV and Fig. 2, where coagulated egg albumin of different sized particles was used. It is evident that the equilibrium

[11] The author does not doubt the existence of adsorption or concentration in the surface layer in the sense of Willard Gibbs. This phenomenon, however, is evidently of subordinate importance here.

reached is independent of the size of the particles and therefore of their surface. These experiments were repeated under slightly different conditions several times—always with the same result. This would indicate that the process is either one of solution, in which

TABLE III.

Removal of Pepsin from Solution by Different Substances.

10 cc. of pepsin solution, pH 2.5, + 0.5 gm. of substances noted. Allowed to stand 10 min. at 25°C. and pepsin estimated in 1 cc. of solution.

Substance.	Relative quantity of pepsin per cc.
Control; pepsin solution alone	100
Starch	98
$CaSO_4$	86
Agar	103
Kaolin	100
Blood charcoal	15
Casein	70
" (coagulated, dried, and ground to 40 mesh)	74
" (extracted with boiling alcohol for 24 hrs.)	60
" C (charred at 150°C.)	90
Egg albumin (coagulated, dried with acetone, and finely powdered)	10

TABLE IV.

Effect of Size of Particles of Egg Albumin, Coagulated, Dried, and Ground.

2.0 gm. in 20 cc. of pepsin solution titrated to pH 2.5 + HCl.
1.0 cc. pipetted off and analyzed for pepsin at time noted.

Time.	Size of particles.	
	Through 10 mesh but not through 20 mesh.	Through 80 mesh.
	Relative amount of pepsin per cc.	
min.		
1		46
2	98	21
4	93	30
8	72	12
30	32	10
60	12	14
120	10	11

case the law of partition coefficients should be found to hold, or of chemical combination, in which case the law of mass action should apply. Preliminary experiments indicate that the process follows the law of partition coefficients.

It may be objected that the effective surface is not the actual surface of the particles but some fine interior structure which is the same in all. The particles, however, swell in acid and appear translucent and homogeneous. It would seem that any interior surfaces must be of nearly intermolecular dimensions. In this case, of course, all chemical phenomena may be considered "surface" phenomena.

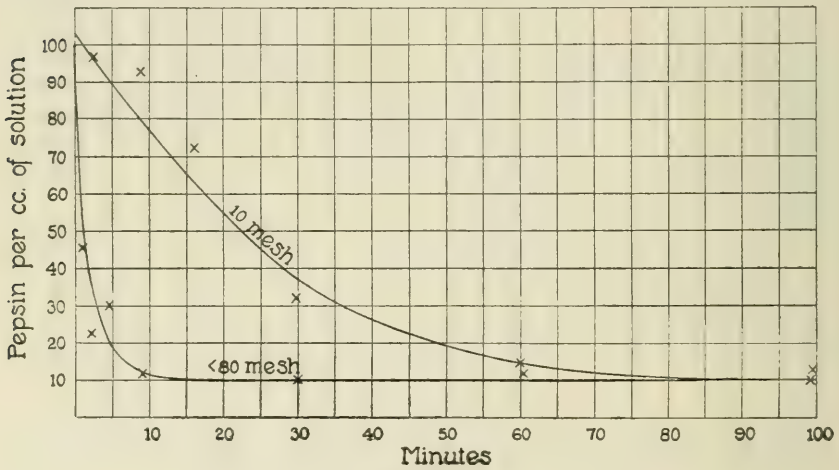

FIG. 2.

II. The Effect of the Hydrogen Ion Concentration.

Inasmuch as the activity of pepsin is dependent to a large extent on the hydrogen ion concentration it appeared probable that the combination of pepsin with its substrate would be a function of the same variable. Table V and Fig. 3 show the results of a series of experiments made at different hydrogen ion concentrations. There is a decided optimum for the combination of pepsin with its substrate corresponding (within the limits of error) to the optimum for digestion. These experiments were repeated with casein with approximately the same results. The optimum zone for the digestion of proteins

by pepsin therefore is due to the fact that at this degree of acidity more pepsin combines with the protein than in either a more or less acid solution. Van Slyke and Zacharias,[12] from a study of the constants of their equation for the action of urease, decided that the hydrogen ion concentration affected the *rate* of combination of the

TABLE V.

Effect of Reaction of Solution on Combination of Pepsin and Coagulated Egg Albumin.

Experiment A.

Temperature 25°C.

0.5 gm. of egg albumin suspended in 10 cc. of HCl of increasing strength. 1.0 cc. of strong pepsin solution added. Tube shaken, allowed to stand 1 min., and clear liquid pipetted off. pH measured (by gas chain) in part of this sample. 5.0 cc. of remainder brought to same reaction in all tubes by addition of a few drops of strong HCl. All brought to same volume with water and pepsin estimated in 1 cc.

Experiment B.

Same as A, but allowed to stand 2 min.

pH of solution.	Relative amount of pepsin per cc.		No albumin. (Control.)	Relative amount of pepsin combined.	
	Experiment A.	Experiment B.		Experiment A.	Experiment B.
0.88	77		100	23	
1.00		79			21
1.24	69			31	
1.3		68			32
2.08	60		100	40	
2.2		50			50
2.75	63			37	
2.8		47			53
3.5	64			36	
3.9		68			32
4.4	77	80		23	20
4.9	100	86	100	0	14
5.7	100	94	100	0	6

enzyme and substrate. In the case of pepsin, however, it is not the *rate* of combination but the *amount* which is influenced. This is shown by the fact that little or no pepsin is removed from its solution by its substrate at a reaction of 5.0, no matter how long they are

[12] Van Slyke, D. D., and Zacharias, G., *J. Biol. Chem.*, 1914, xix, 181.

left in contact. It seems probable that this is due to a change in the condition of the protein rather than to a change in the enzyme, since, according to Ringer,[13] the optimum reaction is different for different proteins.

The simplest explanation of the above facts would seem to be that the quantity of ionized protein present determines the amount of pepsin which combines with the protein, and hence also determines the rate of digestion. Some direct evidence confirming this hypothesis has been obtained and will be discussed fully later.

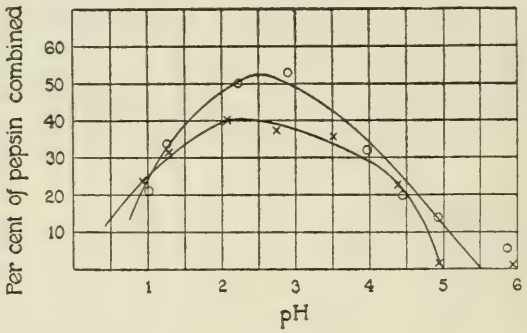

FIG. 3.

SUMMARY.

1. A quantitative method for the determination of pepsin is described depending on the change in conductivity of a digesting egg albumin solution.

2. The combination of pepsin with an insoluble substrate has been followed by this method.

3. The amount of pepsin removed from solution by a given weight of substrate is independent of the size of the particles of the substrate.

4. There is an optimum zone of hydrogen ion concentration for the combination of enzyme and substrate corresponding to the optimum for digestion.

5. It is suggested that the pepsin combines largely or entirely with the ionized protein.

[13] Ringer, W. E., *Kolloid-Z.*, 1916, xix, 253.

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Vol. ii, No. 2, pp. 173–200.]

INFLUENCE OF THE CONCENTRATION OF ELECTROLYTES ON THE ELECTRIFICATION AND THE RATE OF DIFFUSION OF WATER THROUGH COLLODION MEMBRANES.

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(Received for publication, September 6, 1919.)

I. Influence of the Concentration of Electrolytes upon the Rate of Diffusion of Positively Charged Particles of Water through Collodion Membranes.

When we fill a collodion flask, as described in a preceding publication,[1] with a solution of a non-electrolyte, *e.g.* cane sugar, grape sugar, or glycerol, and dip the flask into a beaker containing distilled water, the level of the liquid in the flask will rise, as is to be expected on the basis of the gas pressure theory of osmosis. When we close the opening of the collodion flask with a rubber stopper, perforated by a glass tube with a bore of about 2 mm. in diameter serving as manometer, the rate of diffusion of water into the solution can be conveniently followed. Since at the same time sugar will diffuse out of the flask into the surrounding distilled water, the rise of the column of liquid in the manometer will cease after some time (*e.g.* after about 70 minutes at 24°C.) and will be followed by a fall in the level of the liquid, until finally the solutions inside and outside the collodion flask become identical. It is therefore necessary to consider only the initial rise of liquid in the manometer as an indicator for the attractive action of the solution upon water, and in all the figures given in this paper the readings were made 20 minutes after commencement of the experiment. At the beginning of the experiment the level in the manometer was usually about 30 mm. above that of the distilled water in the beaker in order to discover possible cases of negative osmosis. All collodion flasks had practically the same sur-

[1] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 717.

face, being cast inside of the same Erlenmeyer flask, and during the experiment the whole area of the flask was submersed in the beaker. The temperature was always 24°C.

In Fig. 1 the ordinates are the values for the rise in the level of the solution in the glass tube (after the first 20 minutes) which occurred when the collodion flasks filled with different concentrations of cane sugar, grape sugar, or glycerol were dipped into beakers containing distilled water. The abscissæ are the logarithms of the concentration of the sugar solution. The reader will notice that for concentrations below M/64 the curves run practically parallel to the base line while a sharp rise begins at about M/16 or M/8. If the rise is plotted over the concentration (instead of over the logarithms of the concentration) the curve is almost a straight line between concentrations of M/32 and 1 M (Fig. 2), as was to be expected on the basis of the gas pressure theory. When, however, we make the same experiments with solutions of *electrolytes*, separating them from pure water by collodion membranes, a curious phenomenon is observed, which was partly described in the first paper; namely, that at a very low concentration of electrolyte the rate of diffusion of water through the collodion membrane from pure solvent into the solution increases rapidly with increasing concentration and that it reaches a maximum at a comparatively low concentration[1] of the electrolyte. It is easier to follow the facts to be described with the help of the curves given in Fig. 3. The abscissæ are the logarithms of the concentration of the solution, while the ordinates give the height to which the liquid in the manometer has risen in 20 minutes. The reader will notice that the curves for five sodium salts are given—NaCl, Na_2SO_4, Na_2 oxalate, Na_3 citrate, $Na_4Fe(CN)_6$. The solutions must not be acid for this experiment, and the hydrogen ion concentration of the solutions of NaCl, Na_2SO_4, and Na_2 oxalate was almost that of the point of neutrality (pH about 6.0, or between 6.0 and 7.0), while the two other solutions were slightly alkaline. Beginning with the lower concentrations of the solutions of these salts the curves rise sharply with the increase in concentration, reach a maximum at a concentration of about M/256, and then with a further increase in concentration the curves fall abruptly to reach a minimum, varying for the different salts between M/32 and M/8. After this the curves rise again.

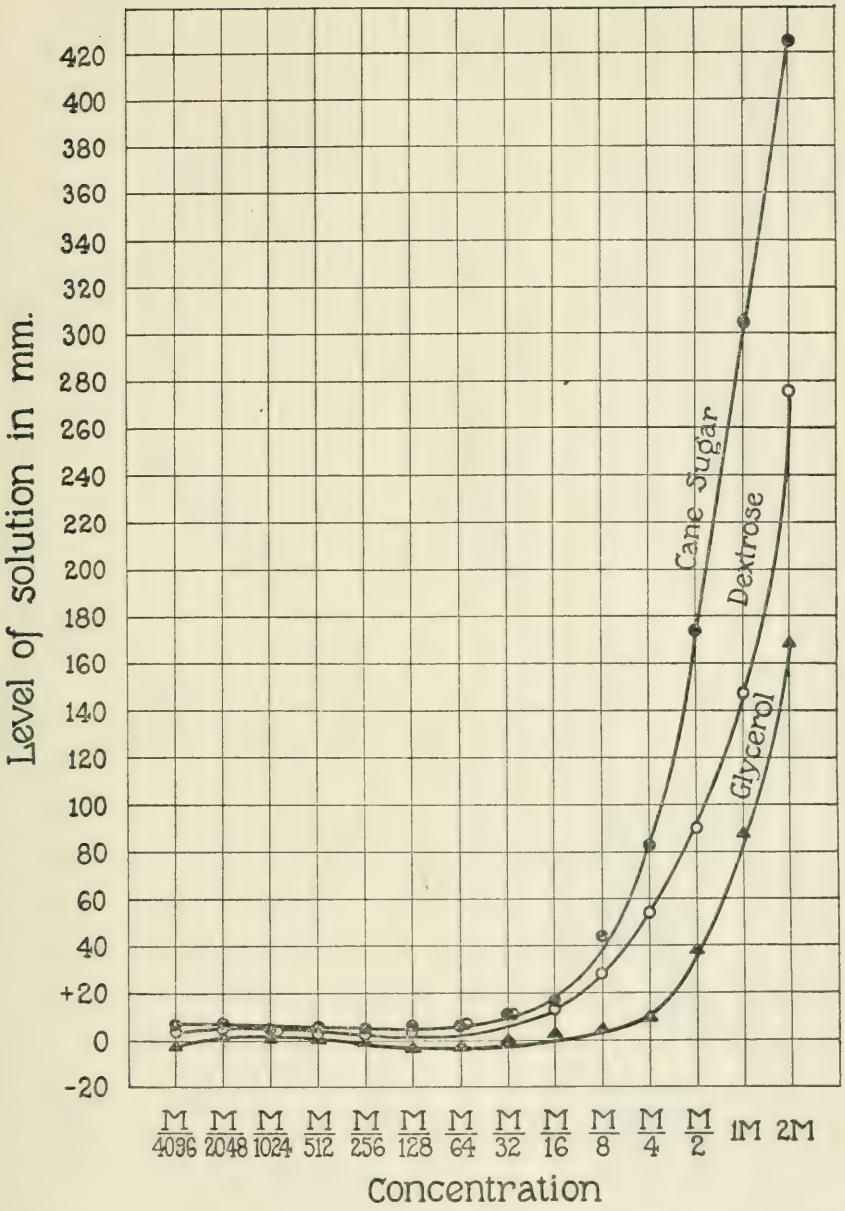

FIG. 1. Influence of concentration of non-electrolytes on initial rate of diffusion of water through a collodion membrane from pure solvent to solution. Abscissæ are logarithms of concentrations, ordinates the rise in mm. in level of solution in the manometer tube after 20 minutes.

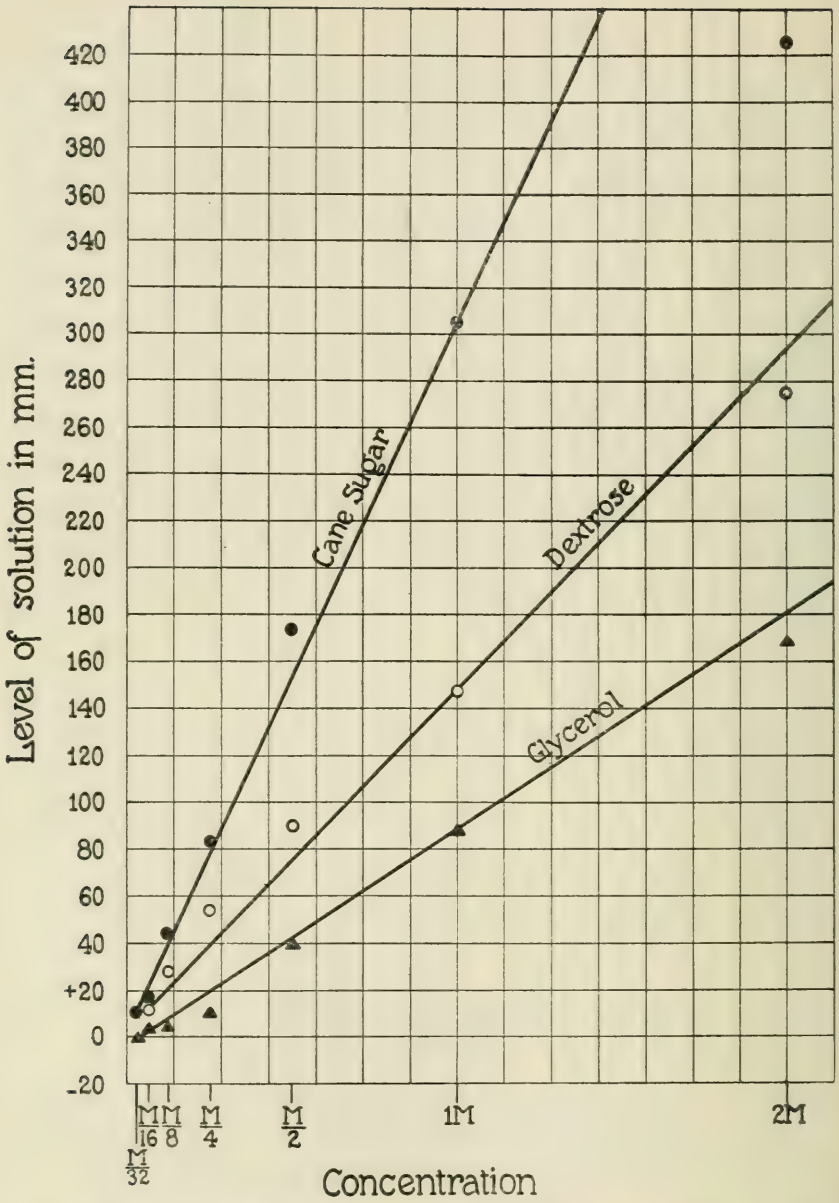

FIG. 2. The same as in Fig. 1, except that abscissæ in Fig. 2 are the concentrations instead of the logarithms of concentrations as in Fig. 1. Within concentrations of M/32 and 1 M the initial rise of liquid in 20 minutes is in direct proportion to the concentration, as van't Hoff's law demands.

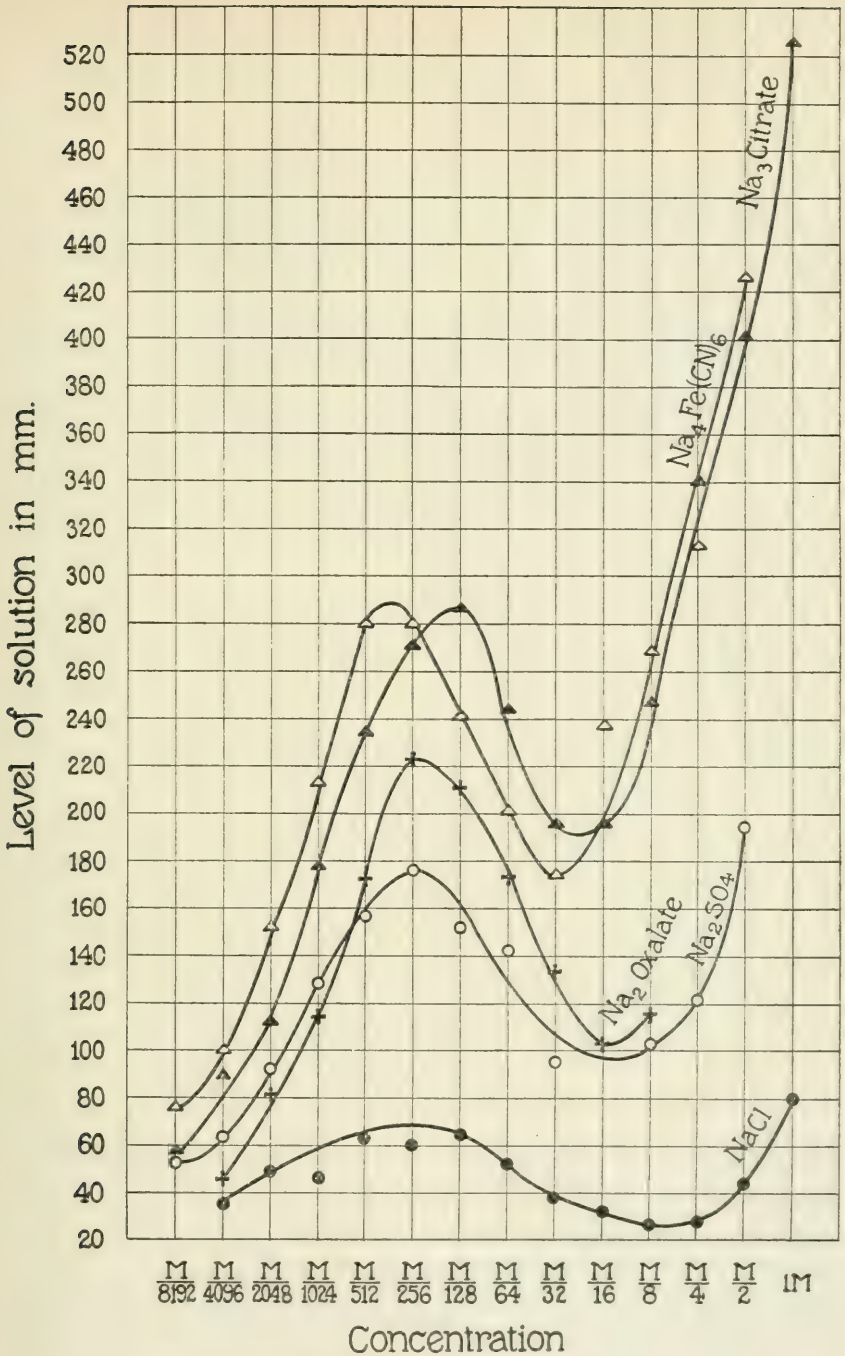

FIG. 3. Curves representing influence of concentration of five different sodium salts upon initial rate of diffusion of water through a collodion membrane from pure solvent to solution. Abscissæ are the logarithms of concentration. Ordinates represent level of solution in manometer tube after 20 minutes. The curves rise steeply until the concentration of M/256 is reached, then, with further increase in concentration, the curves drop, and rise again when the concentration is between M/16 and M/4.

While the ordinates of these curves represent the height in the manometer 20 minutes after the beginning of the experiments, the same form of curves is obtained when the readings are taken after 5, 10, or 30 minutes.

The interpretation of this complicated system of curves becomes simplified if we compare it with the curves for non-electrolytes in Fig. 1. We then notice that the character of both sets of curves agrees in the region of the higher concentrations above from M/32 to M/4, and we are therefore inclined to assume that the second rise in the curves for solutions of sodium salts beginning with M/32 to M/4 (according to the nature of the salt in solution) is due mainly to the gas pressure effect of the solution.

The abrupt rise and fall of the curves in Fig. 3 for lower concentrations than M/32 are not repeated in the case of the curves for the non-electrolytes in Fig. 1, and we are inclined to ascribe these peculiarities of the curves to the electrical action of the ions on the rate of diffusion of the electrified particles of water. Fig. 4 expresses this division of the curves diagrammatically.

In a preceding paper[1] we have shown that in the presence of neutral (or alkaline) salts with monovalent or bivalent cation the particles of water diffuse through the membrane as if they were positively charged, being attracted by the anion of the salt and repelled by the cation with a force which increases with the number of charges of the ion (and inversely with a quantity which we arbitrarily designated as the "radius" of the ion). The source of the electrification of the water particles as well as the specific mechanism by which the ions of the solution influence the rate of diffusion of water will not be discussed in this paper. It will, however, simplify the presentation of our experiments if it be permitted to describe our results as if the charged water particles were attracted or repelled electrostatically by the ions of the solution. We notice that in Fig. 3 the curves rise the more rapidly with increasing concentration the higher the valency of the anion of the salt, and with the reservation just mentioned we may ascribe this initial steep rise in the curves between a concentration of the solution of 0 to a concentration of M/256 to the fact that the attractive action of the anion of the sodium salts upon the rate of diffusion of the positively charged particles of water through the mem-

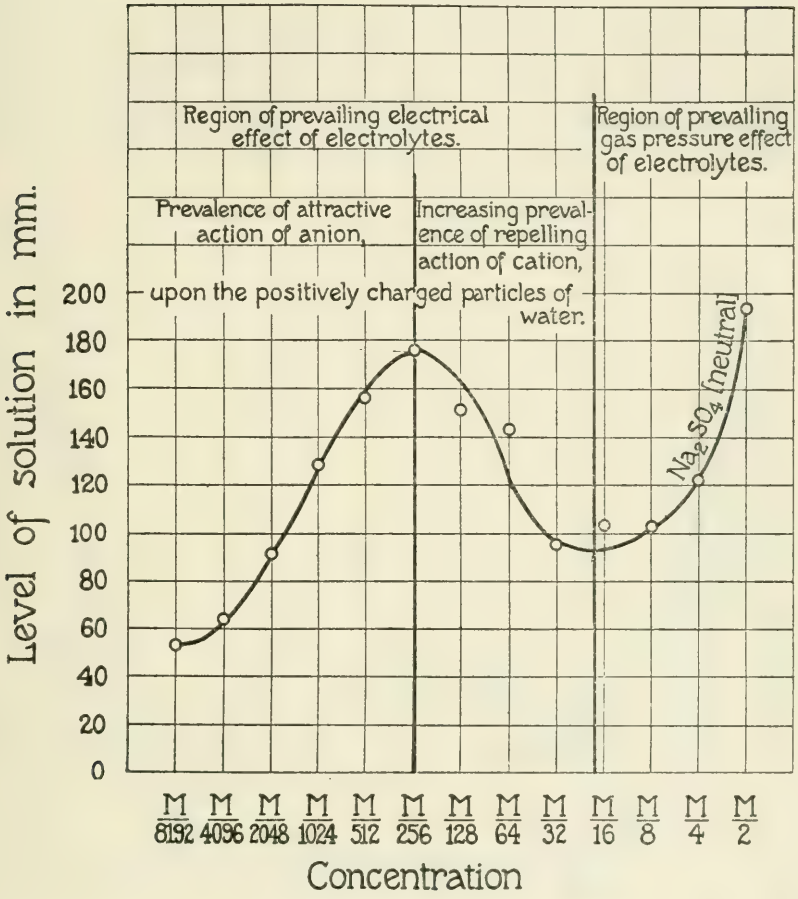

FIG. 4. Explanation of the curves in Fig. 3 given for the Na_2SO_4 curve. The first rise and fall between M/8,192 and M/16 represent the electrostatic action of ions of the solute upon the rate of diffusion of positively charged water particles. The second rise beyond M/16 represents the rise due to the gas pressure effect of the solute. The steep rise of the curve between 0 and M/256 is due to the prevalence of the attractive action of anion upon the positively charged molecules of water, while the drop beyond M/256 is due to the fact that the repelling action of cation upon the positively charged particles of water increases more rapidly with increasing concentration than the attractive action of SO_4 for the water.

brane is greater than the repelling action of the cation. The curves reach a maximum when the solution reaches a molecular concentration of about M/256 and from now on the curves drop rapidly with a further increase in the concentration of the solution. We assume that when the concentration of the solution of the sodium salts reaches a certain value the repelling action of the cation—in this case Na—

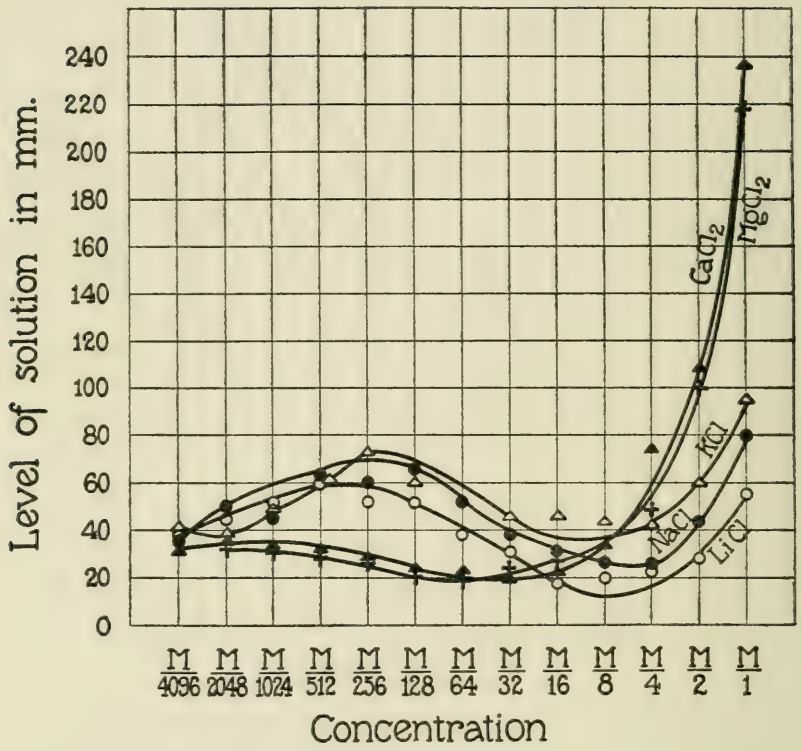

FIG. 5.

increases more rapidly with increasing concentration than the attractive action of the anion upon the positively charged particles of water. This drop, however, is comparatively smaller the higher the valency of the anion. In the case of sodium chloride (Fig. 3) the curve is lower at M/8 (the minimum) than at M/4,096, while for SO_4 and oxalate the minimum reached by the curves (at M/16) is considerably above that

reached by the NaCl curve, and the minimum for the citrate and ferrocyanide is still higher. This was to be expected from the fact that the attractive action of the anion upon the positively charged particles of water is higher the greater the number of charges or the valency of the anion.

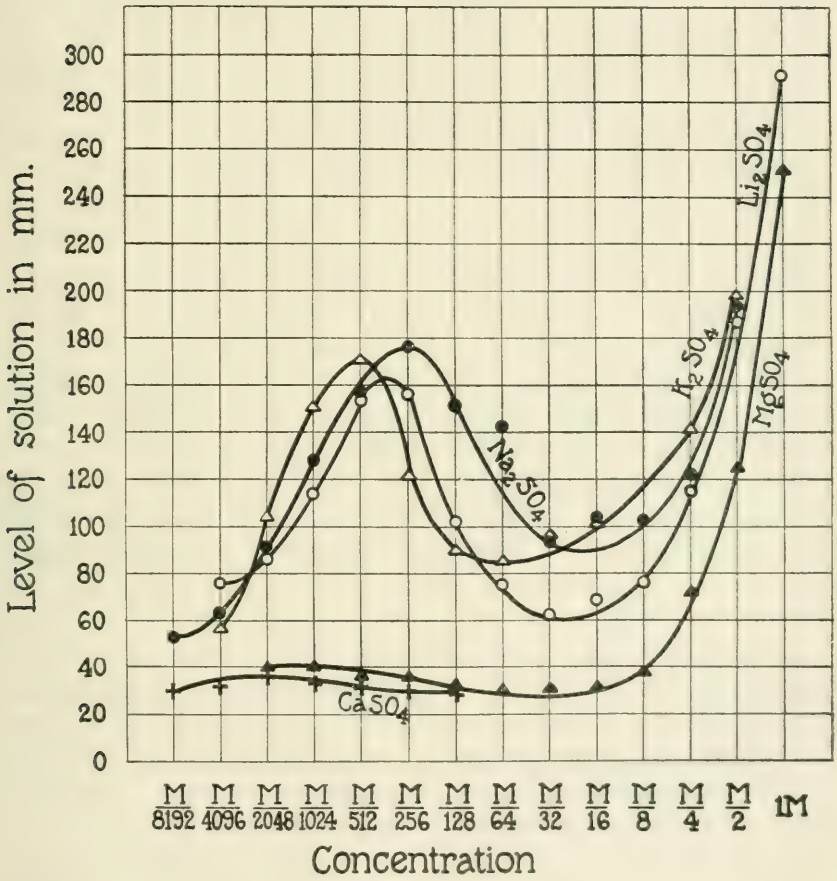

FIG. 6.

FIGS. 5 and 6. Curves representing influence of different chlorides (Fig. 5) and sulfates (Fig. 6) on initial rate of diffusion of water from pure solvent into solution, showing that the drop is greater in case of Li than of Na, or of K, and supporting the view that the drop is due to the repelling effect of the cation upon the positively charged particles of water.

The facts given are not peculiar to sodium salts but can be demonstrated in the case of all neutral or alkaline solutions of salts with monovalent or bivalent cation. Fig. 5 gives the curves for different chlorides and Fig. 6 for different sulfates. All the curves for these salts rise up to a concentration of about M/256 and then fall and finally rise again when the concentration is so high that the gas pressure effect begins to obliterate the electrical effect of the ions. The turning point for the commencement of the prevalence of the gas pressure effect is at a lower gram-molecular concentration for the sulfates than for the chlorides; Fig. 3 shows that the concentration of the turning point is the lower the higher the valency of the anion. It may be that this is simply the consequence of the fact that the higher the valency of the anion the greater the number of ions into which the salt dissociates electrolytically, but it is not yet certain that this is the full explanation. Another fact, however, is certain; namely, that the depression of the curves when the concentration exceeds M/256 is greater for the lithium salts than for the sodium salts, and greater for the sodium salts than for the potassium salts. This was to be expected if the drop is due to the repelling action of the cation on the positively charged particles of water, since the repelling action of cations on the positively charged particles of water increases inversely with a quantity which we arbitrarily designate as the "ionic radius." This "ionic radius" is less for Li than for Na and presumably less for Na than for K.

In the case of neutral solutions of salts with bivalent cation the repelling action of the cation is so great that it balances or exceeds the initial attractive action of a bivalent or monovalent anion for positively charged particles of water; the depressing action of the cation in concentrations above M/256 upon positively charged particles of water remains, however, noticeable especially in the SO_4 curves.

II. Influence of the Concentration of Electrolytes upon the Rate of Diffusion of Negatively Charged Particles of Water through Collodion Membranes.

The curves given in Figs. 3 to 6 contain a paradoxical fact, which can be expressed as follows: *when solutions of neutral or alkaline salts with monovalent or bivalent cation are separated by a collodion membrane*

from pure water the attraction of the solution for water diminishes with increasing concentration for concentrations between M/256 *to about* M/32 *or above, according to the nature of the electrolyte.* This is exactly the reverse of what we should expect on the basis of van't Hoff's law which demands that the attraction of the solution for water should increase with the concentration. Such a reversal occurs only in the case of electrolytes and we assume provisionally that it is due to the fact that the repelling action of the cation of the electrolyte upon the positively charged particles of water increases inside the critical range of concentrations more rapidly with the concentration than the attractive action of the anion upon the same particles of water.

It will be necessary to test this assumption by further experiments.

We have shown in the first paper that water particles diffuse through collodion membranes in the form of *positively* charged particles from pure solvent to solutions of electrolytes with monovalent or bivalent cation when the solution is neutral or alkaline in reaction. When, however, the solution is rendered sufficiently acid the water particles diffuse through the membrane as if they were *negatively* charged, being attracted by the cation and repelled by the anion of the electrolyte with a force increasing with the valency of the ions. We will now consider the influence of the concentration of electrolytes upon the rate of diffusion of *negatively* charged particles of water through the membrane.

We have stated in a former paper[2] that the collodion membranes were suspended for 1 night in a 1 per cent gelatin solution before being used; after this they were used sometimes for a week or more without any further gelatin treatment. The membranes were, of course, rinsed a large number of times in warm water after the gelatin treatment to remove all the gelatin that could be removed by rinsing. This gelatin treatment is not necessary to obtain the results with neutral solutions of electrolytes described in the preceding chapter. The gelatin treatment of the membrane is, however, necessary to obtain the results with negatively charged particles of water to be described in this chapter. We intend to return to this fact in a later paper.

[2] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 87.

Negatively charged particles of water are attracted powerfully by bivalent or polyvalent cations and repelled powerfully by bivalent or polyvalent anions. We may, therefore, expect that when we make a $CaCl_2$ solution sufficiently acid through the addition of HNO_3 or HCl the curves showing the influence of the concentration of the electrolyte on the rate of diffusion of water should be similar to the curves in Figs. 3, 4, and 6, representing the influence of different concentrations of neutral solutions of Na_2SO_4 upon the rate of diffusion of positively charged particles of water through the same membranes. Fig. 7 shows that this is correct. The two upper curves represent the influence of variations in the concentration of $CaCl_2$ or $Ca(NO_3)_2$ dissolved in M/1,024 or M/128 HNO_3 or HCl. These acid solutions of $CaCl_2$ or $Ca(NO_3)_2$ cause a negative electrification of the water particles diffusing through the membrane. The attraction of the negatively charged water particles by Ca ions increases with an increase in the concentration of the calcium salt up to M/64, when the curve falls until the concentration of the calcium salt is M/4 where a minimum is reached. After this the gas pressure effect makes itself felt and the curve rises rapidly with rising concentration. In this case the drop in the two curves for the acid solutions when the concentration of the calcium salt exceeds M/64 is due to the fact that beyond a certain concentration of the calcium salt the repelling effect of the anion (Cl and NO_3) upon the negatively charged particles of water increases more rapidly with increasing concentration than the attractive action of the Ca ion.

The fact that the curve for $Ca(NO_3)_2$ in M/128 HCl is slightly lower than the curve for $CaCl_2$ in M/1,024 HNO_3 is due to the "negative osmosis" caused by acid solutions of sufficient concentration, as will be shown in the next chapter.

The three lower curves of Fig. 7 represent the influence of various concentrations of $CaCl_2$ upon positively charged particles of water and these curves are similar to those given for the effect of neutral solutions of $CaCl_2$ in Figs. 5 and 6. When $CaCl_2$ is dissolved in M/128 KOH the rise and drop of the curve become a little more marked than when the solution of $CaCl_2$ is neutral. The reason for this will become clear through the discussion in the next chapter of the phenomenon of negative osmosis caused by $Ca(OH)_2$.

When we render Na_2SO_4 solutions acid (by dissolving the salt in M/128 or M/1,024 HNO_3) the negatively charged particles of water diffusing through the membrane will be repelled by the SO_4 ion and attracted by both the Na and H ions. Since the SO_4 ion carries two

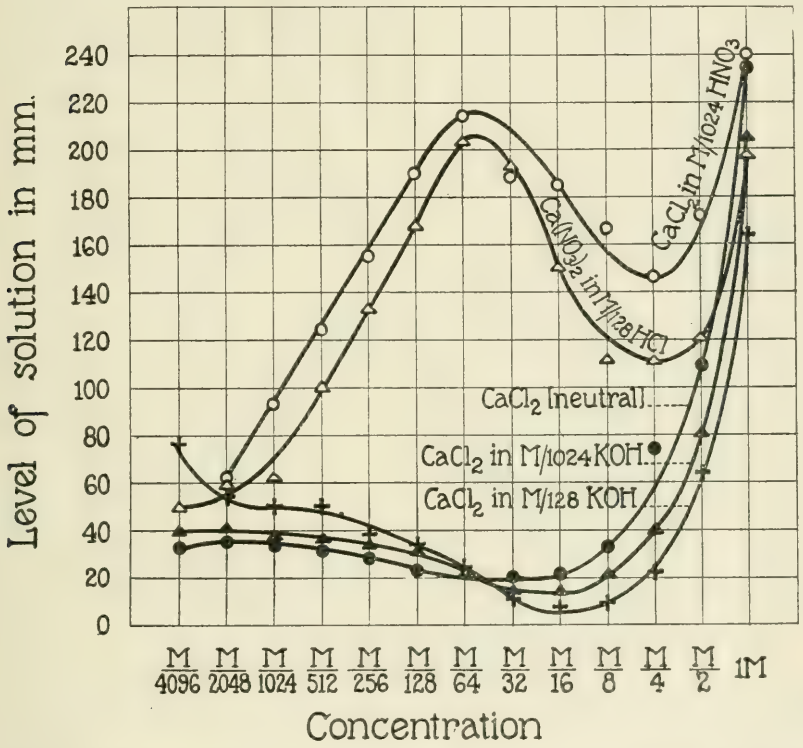

FIG. 7. Upper two curves representing influence of acid solutions of $CaCl_2$ or of $Ca(NO_3)_2$ upon negatively charged particles of water. At first there is a steep rise of the curve due to prevalence of attraction of Ca for the negatively charged particles of water, then beyond M/64 a drop in the curve due to prevalence of repelling action of NO_3 and Cl upon the negatively charged particles of water. No initial rise in the three lower curves, since in neutral and alkaline solutions of Ca water diffuses in the form of positively charged particles which are too strongly repelled by Ca to permit an initial rise in the level of the solution.

charges while the cations Na and H are monovalent the greater electrostatic action of the SO_4 ion should inhibit the electrostatic attraction of water by such solutions in the same way as is done by neutral so-

lutions of $CaCl_2$ in the case of positively charged particles of water. Fig. 8 shows that this is the case. The two lower curves in Fig. 8 show the influence of concentrations of sufficiently acid solutions of Na_2SO_4 upon the rate of diffusion of water from distilled water into

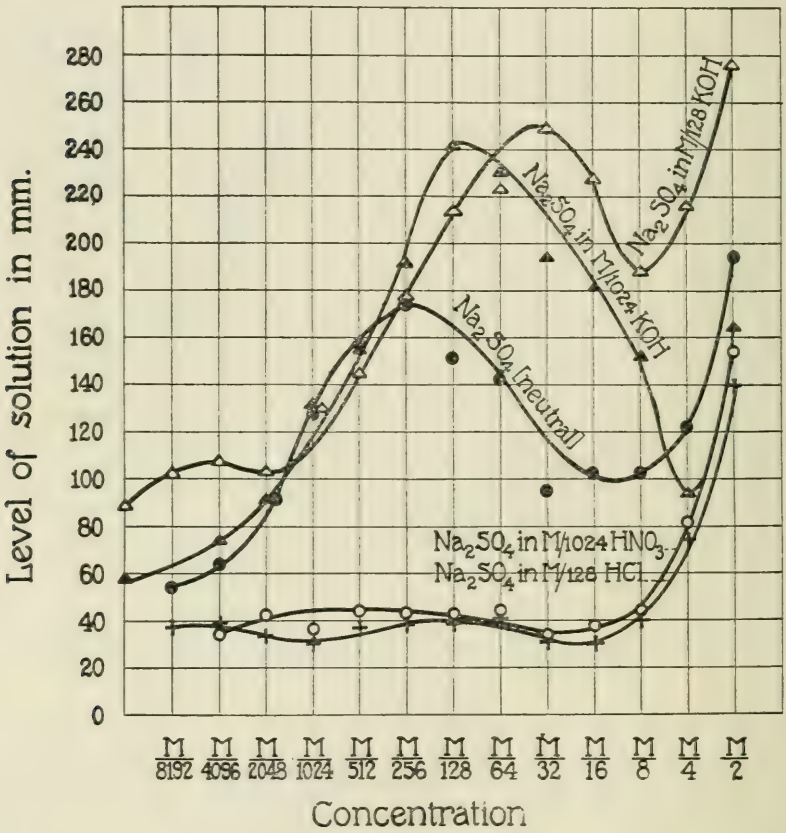

FIG. 8. The curves for solutions of Na_2SO_4 when sufficiently acid show no initial rise, since the negatively charged particles of water are too strongly repelled by the SO_4 ions.

solution. The curves resemble the curves for the effect of different concentrations of non-electrolytes, except for an intimation of a slight drop for concentrations of Na_2SO_4 between M/64 and M/8. The three upper curves represent the influence of concentrations of different

neutral and alkaline solutions of Na_2SO_4 upon the rate of diffusion of positively charged water particles.

It was finally expected that the curves representing the influence of various concentrations of NaCl upon the rate of diffusion of water through collodion membranes should be similar in neutral, acid, and alkali solutions, for the reason that anion and cation of the salt are

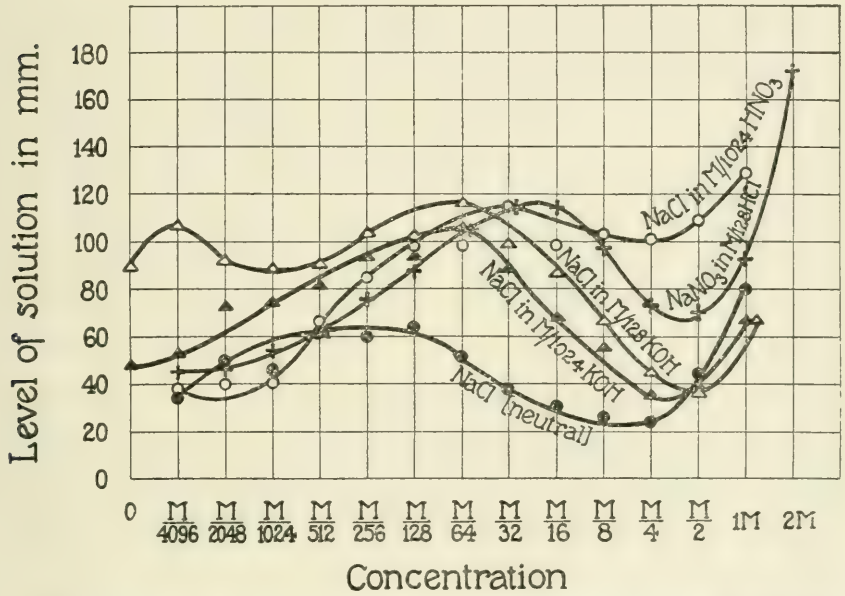

FIG. 9. Salts with monovalent anion and cation, like NaCl, show both rise and drop in alkaline, neutral, and acid solutions. In neutral and alkaline solutions the first rise is due to the attractive action of the Cl ion and the drop to the repelling action of the Na ion upon the positively charged particles of water. In acid solution the first rise in the curve is due to the attractive action of the Na ion and the drop to the repelling action of the Cl ion upon the negatively charged particles of water.

both monovalent (Fig. 9). In the presence of a sufficiently acid solution of NaCl the particles of water diffuse as negatively charged molecules through the membrane which are attracted by the Na ions and repelled by the Cl ions. The initial rise of the curve is due to the attractive action of the Na ion, and the drop in the curve is due to the increasing prevalence of the repelling action of the Cl ion. In neutral

and alkaline solutions of NaCl the Cl ion attracts the water (which is charged positively) and the fall of the curve is due to the Na ion. The complete suppression of the rise which is caused in $CaCl_2$ when the solution is neutral or alkaline, and in Na_2SO_4 when the solution is acid, is due to the fact that in these cases the attracting ion is mono-

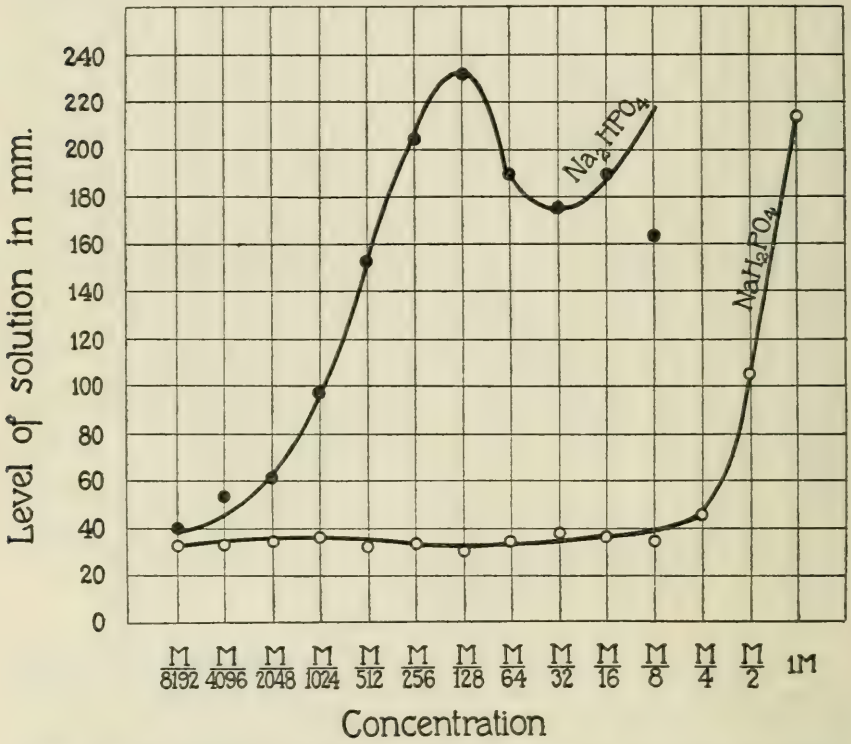

FIG. 10. The Na_2HPO_4 curve shows the initial rise and drop since the water diffuses in the presence of this salt in the form of positively charged particles which are attracted by the trivalent PO_4 ion and repelled by the monovalent Na and H ions. The NaH_2PO_4 curve shows no initial rise, since in the presence of this salt water diffuses in the form of negatively charged particles which are repelled by the trivalent PO_4 ion and attracted by the monovalent Na and H ion.

valent and the repelling ion bivalent. Fig. 9 shows that the curves representing the influence of the concentration of NaCl solutions upon the rate of diffusion of water are similar for neutral, alkaline, and acid solutions of NaCl. The drop is more considerable for $NaNO_3$ solu-

tions in M/128 HCl than in M/1,024 acid, on account of the negative osmosis caused by solutions of acids, as the next chapter will show.

Fig. 10 shows the difference in the influence of concentration upon the rate of diffusion of water in the case of Na_2HPO_4 and NaH_2PO_4. In the presence of the latter solution the water diffusing through the membrane is negatively charged and the curve does not show the initial rise and drop but shows only the gas pressure effect of the solution. In the case of Na_2HPO_4 water diffuses in the form of positively charged particles and the curve shows the rise and drop as described for neutral and alkaline salts in which the anion has a higher valency than the cation.

We can therefore state that for the diffusion of negatively as well as positively electrified particles of water the law of van't Hoff is reversed within a certain range of concentrations of electrolyte—in the extreme between about M/256 and M/4—inasmuch as in this range the attraction of a solution of an electrolyte for pure water diminishes with increasing concentration.

III. Negative Osmosis.

Dutrochet,[3] Graham,[4] and Flusin[5] have observed a striking phenomenon of negative osmosis in certain acids; namely, that the stream of liquid diffuses through pig's bladder from acid to pure water, instead of in the opposite direction. This was observed for tartaric and oxalic acids. Flusin tries to explain the phenomenon on the basis of a difference in degree of imbibition or swelling of the two sides of pig's bladder, one side being in contact with pure water where the swelling is slight, the other being in contact with acid where the swelling is great. Bartell, and Bartell and Hocker have observed negative osmosis through porcelain even in the case of some neutral

[3] Dutrochet, H., *Ann. chim. et. phys.* 1835, lx, 337.

[4] Graham, T., *Phil. Tr.*, 1854, cxliv, 177.

[5] Flusin, G., *Ann. chim. et phys.*, 1908, xiii, 480. For a discussion of the literature see Höber, R., Physikalische Chemie der Zelle und der Gewebe, Leipsic, 4th edition, 1914, 248.

[6] Bartell, F. E., *J. Am. Chem. Soc.*, 1914, xxxvi, 646. Bartell, F. E., and Hocker, C. D., *J. Am. Chem. Soc.*, 1916, xxxviii, 1029, 1036.

salts. The writer has never observed negative osmosis when solutions of neutral salts were separated from pure solvent by collodion membranes.

The writer has recently investigated the influence of the concentration in acids and alkalies on the rate of diffusion of water through collodion membranes (previously treated with gelatin), with the result that the phenomenon of negative osmosis described for tartaric and oxalic acids is very common in acids and alkalies, that it *occurs in exactly the same range of concentration where the drop in the curves of the neutral salt solutions occurs, namely within a range between* M/256 *to about* M/4, *and that the phenomenon is in reality nothing else but this drop.* The difference between the nature of the drop in the case of solutions of neutral salts on the one hand and of solutions of acids and alkalies on the other is that in the case of alkalies and acids the drop is not only relative but absolute. Titration experiments show that acid diffuses from the acid solution into distilled water and that the concentration of acid in the solution is considerably less after 20 minutes than at the beginning. Since at the same time the total volume of solution diminishes—this being the nature of negative osmosis—we must conclude that the diminution of the volume of the solution is due to the fact that the combined volume of acid and water diffusing out from the solution is slightly larger than the volume of water diffusing simultaneously into the solution. We shall return to this problem in a subsequent paper. Since acids as well as bases diffuse into the pure solvent the phenomenon of negative osmosis can only be observed during a short period at the beginning of the experiment.

In all the experiments described in the preceding pages it was necessary to put the solution into the collodion flask and to dip the latter into distilled water in order to observe the influence of the solution on the initial rate of diffusion of water. More water diffuses into the solution than diffuses simultaneously in the opposite direction and as a consequence the level of the water in the glass tube rises. When we put solutions of certain acids or alkalies into the collodion flasks and dip these flasks into distilled water the level of the liquid in the manometer falls instead of rising. If, however, we put the solution of the same acids or alkalies outside, filling the collodion flask with

distilled water, we observe an initial rise of liquid in the manometer. For these experiments with acids, the collodion membranes must be bathed once over night in a 1 per cent gelatin solution. Fig. 11 shows the influence of the nature and concentration of acid upon the phenomenon. Since we are dealing here with a loss of volume on the

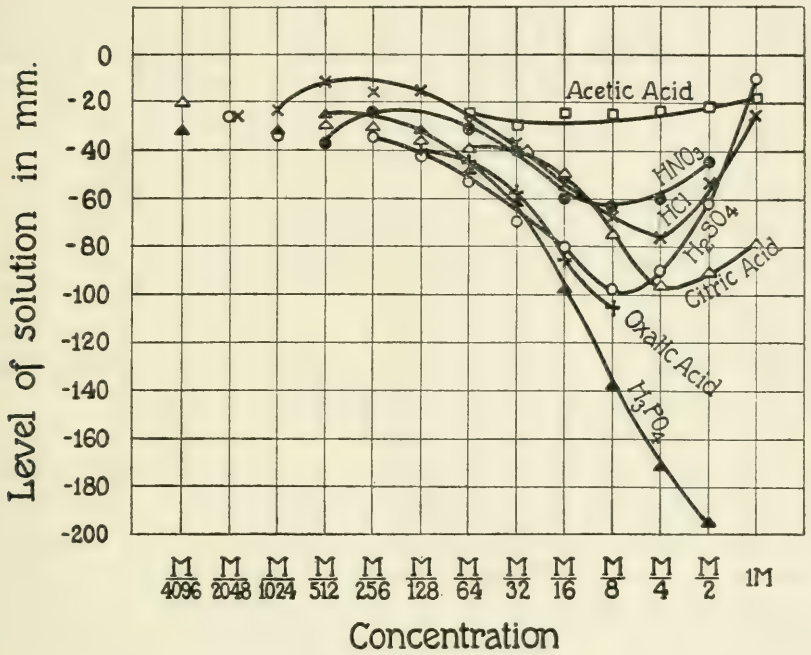

FIG. 11. Negative osmosis in acids, showing that the drop occurs here as in all other cases in a range of concentrations between M/256 and M/8 or M/4. The drop is greater for acids with bivalent or trivalent anion than for acids with monovalent anion, showing that the drop is due to the repelling action of the anion upon the negatively charged particles of water.

part of the solution, we must plot the rise observed in the manometer as a negative quantity below the zero line. The curves show that the drop is confined to the same range of concentrations where a similar drop occurs in the case of the neutral salts. The level of the water in the manometer tube was at the beginning of the experiment about 30 mm. When the flasks thus filled with distilled water were put

into beakers containing solutions of acid the level of the pure water in the flask rose during the first 20 minutes or more whenever the concentration of the acid was M/256 and the rise increased with an increase in the concentration until the concentration was about M/4. Then the level of the water inside the flask fell again.

In the presence of acids water diffuses through the membrane in the form of negatively charged particles. We have assumed that the drop is due to the repelling action of that ion of the electrolyte which has the same charge as the water particles; *i.e.*, the anion in the case of acids. Our assumption is supported by the fact that aside from the degree of electrolytic dissociation the drop increases with the increase of valency of the anion of the acid as we should expect. The drop is least in the case of the acids with monovalent anion (NO_3 and Cl), is greater in the case of SO_4 and oxalate anion, and still greater in the case of PO_4. The drop in citric acid is a little less than in oxalic acid, as is to be expected from the fact that citric acid is a weak acid. In the case of acetic acid no rise and drop in the curve is noticeable except the rise due to the gas pressure effect of the solution.

In the case of alkalies we are restricted to low concentrations by the fact that they dissolve the collodion membranes when the concentration becomes moderately strong. As Fig. 12 shows, we observe absolute negative osmosis in the case of $Ca(OH)_2$ and $Ba(OH)_2$ when the concentration of the solution exceeds M/256. In the case of alkalies with monovalent cation the usual drop in the curves—*i.e.* relative negative osmosis—was observed, but not an absolute negative osmosis; *i.e.*, an absolute diminution of the volume of the solution.

The negative osmosis in the case of acids and alkalies becomes absolute for the reason that the initial rise in the curve due to the attractive action of the ion with the opposite sign of charge from that of the water is too slight or entirely lacking in the case of the acids or alkalies.

The writer was interested in finding out whether aluminium salts with bivalent or trivalent anion induce negative osmosis. In Fig. 13 are plotted the curves representing the influence of different concentrations of Al_2Cl_6, $Al_2(SO_4)_3$, and aluminium citrate on the rate of diffusion of water through a collodion membrane. All the solutions had about the same hydrogen ion concentration and the water diffused

through the membrane in the form of negatively charged molecules, which according to our theory are attracted by the Al ion and repelled by the anion, the repelling action increasing with the valency of the anion. The curves show that Al_2Cl_6 attracts water very violently, $Al_2(SO_4)_3$ much less, and that aluminium citrate acts like a solution of a non-electrolyte, the attractive and repelling action of the two trivalent ions, Al and citrate, balancing each other. But no negative osmosis was noticed. This shows that the electrostatic effect of the H ion upon the rate of diffusion of water is considerably smaller than

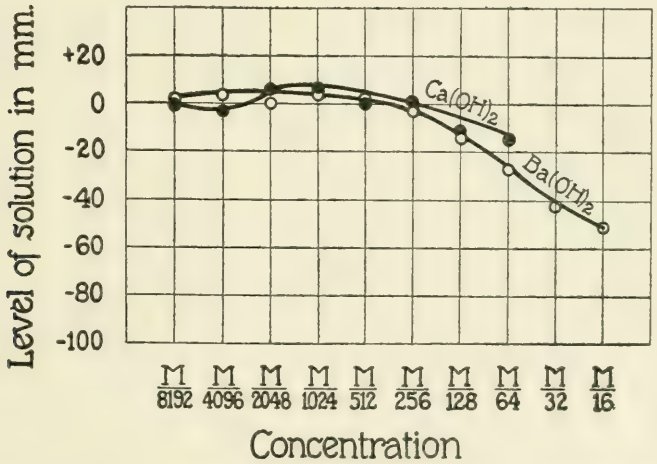

FIG. 12. Curves showing negative osmosis in the case of $Ca(OH)_2$ and $Ba(OH)_2$ occurring in that range of concentrations where the drop in Fig. 3 occurs.

the electrostatic action of trivalent cations like Al, while the electrifying effect of the H ion upon the water seems to be more nearly equal to the electrifying effect of the trivalent cation upon water. This suggests that the electrifying effect of ions upon water and their electrostatic effect upon the rate of diffusion of water are due to different qualities of the ion.

The curves for aluminium salts in Fig. 13 show no drop. As a matter of fact, a slight drop can be demonstrated in the $AlCl_3$ curves but in concentrations higher than those in Fig. 13, namely about M/32 or M/16, a phenomenon which finds its explanation perhaps in the fact that the cation is trivalent and the anion only monovalent.

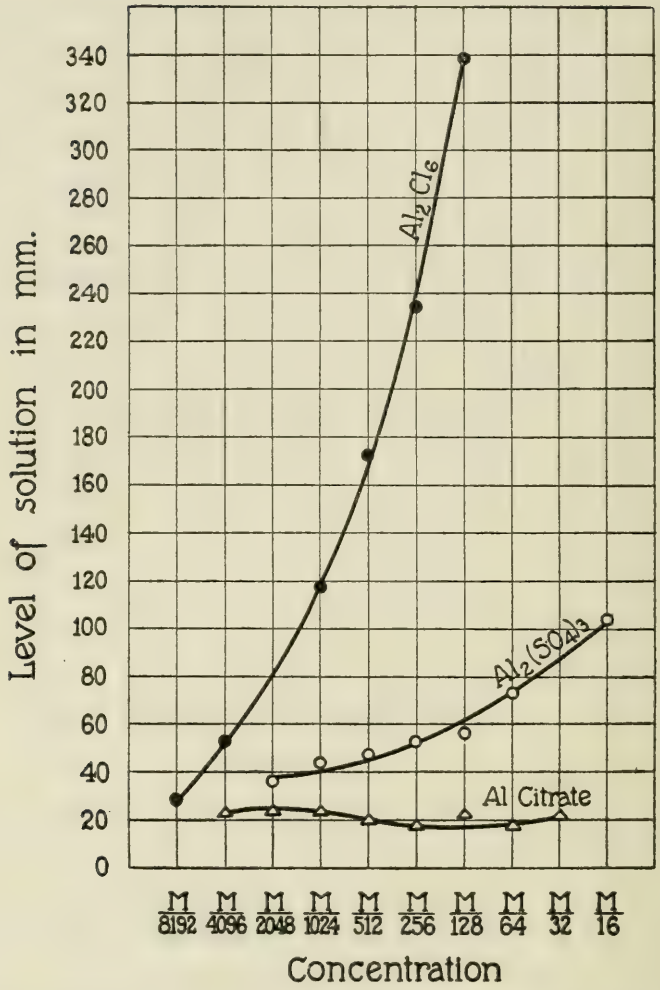

FIG. 13. Curves representing the attraction of different aluminium salts for negatively charged particles of water. The attraction is a maximum in the case of Al_2Cl_6, is less in the case of $Al_2(SO_4)_3$, and is practically lacking in the case of aluminium citrate, showing the increase in the repelling effect of the anion upon the negatively charged particles of water with increasing valency of the anion.

IV. Further Proof that the Negative Osmosis Is Due to the Repelling Action of the Ion with the Same Sign of Charge as that of the Electrified Water Particles.

When we fill the collodion flask with a M/256 solution of Na_2SO_4 (made neutral or slightly alkaline) and dip the flask into distilled water, we notice a rather high initial rate of diffusion of water into the flask caused by the fact that the attraction of the SO_4 ion with its two charges upon the positively charged particles of water is greater than the sum of the repelling action of the two Na ions. In higher concentrations of Na_2SO_4 this difference becomes less (Fig. 4), as we assume through the fact that for some reason the repelling action of the Na ions on the positively charged water particles increases more rapidly with further increase in concentration than the attractive action of SO_4. If this assumption is correct the addition of a salt of the type $MgCl_2$ to a M/256 solution of Na_2SO_4 should lower the rate of diffusion of water into the M/256 solution of Na_2SO_4 more than the addition of the same concentration of KCl; since the repelling effect of the bivalent Mg ion upon the positively charged water particles is greater than the repelling effect of the monovalent K ions. The addition of LiCl should lower the rate of diffusion of water more than the addition of KCl but less than $MgCl_2$, since the electrostatic effect of Li is greater than that of the K ion but less than that of the Mg ion. Fig. 14 shows that this is actually true. 100 cc. of M/256 Na_2SO_4 (pH about 6.0) contained 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, and 25.6 cc. of M/4 KCl, LiCl, or $MgCl_2$. The addition of KCl caused only a slight diminution in the rate of diffusion when 12.8 to 25.6 cc. of M/4 KCl were added. LiCl caused more of a drop, but a slight addition of $MgCl_2$ caused a considerable drop, as our theory demands.

When, however, a salt with polyvalent anion and monovalent cation like $K_4Fe(CN)_6$ is added to M/256 Na_2SO_4 the attraction of the ferrocyanide ion for water counteracts to a large extent the drop caused by the weak repelling action of the K ions added. Fig. 3 shows that the attraction of the $Fe(CN)_6$ ion for water is greater than that of the sulfate ion. Hence the addition of slight quantities of $K_4Fe(CN)_6$ to M/256 Na_2SO_4 increases the initial rate of diffusion of water into the solution but as soon as the concentration of $K_4Fe(CN)_6$ added reaches

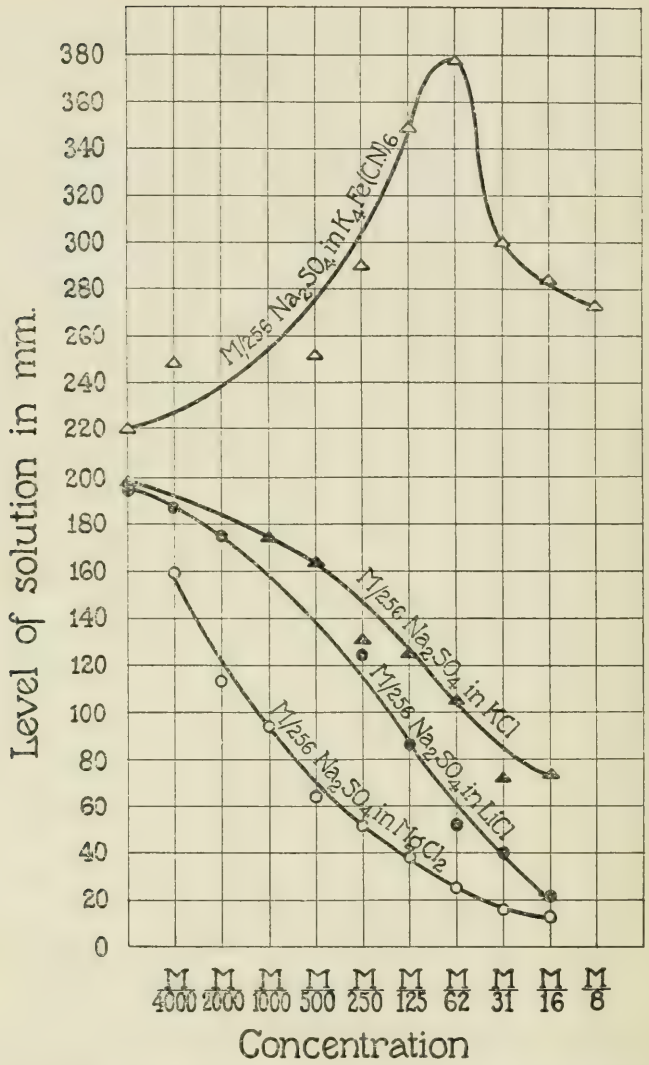

FIG. 14. Curves showing that when we add increasing concentrations of neutral salts with monovalent anion to a neutral M/256 solution of Na_2SO_4 the attraction of the Na_2SO_4 solution for water is diminished. Since this diminution increases in the order K, Li, Mg, the latter being the most depressing ion, it supports the idea that the drop observed in curves of Fig. 3 is due to the repelling action of the cation upon the positively charged particles of water.

the concentration of M/62 a further addition of $K_4Fe(CN)_6$ lowers the rate of diffusion of water into the solution. From this concentration on the repelling action of the K and Na ions upon water prevails over the attractive action of the anion.

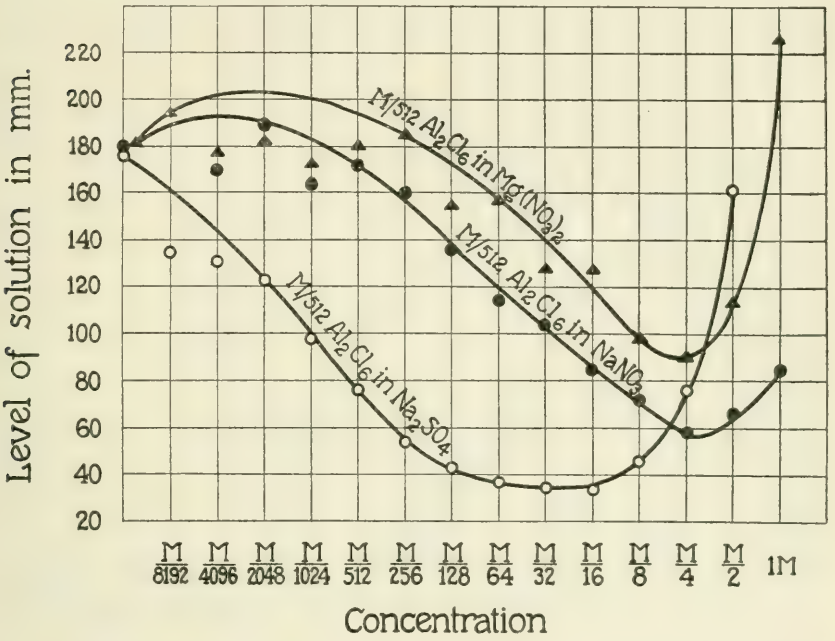

FIG. 15. Curves showing that when we add increasing concentrations of $NaNO_3$, $Mg(NO_3)_2$, and Na_2SO_4 to M/512 solutions of Al_2Cl_6 the depressing effect is greater for SO_4 than for NO_3. Since the water diffuses in this case in the form of negatively charged particles the curves support the idea that the depressing effect of the salt added is due to the repelling action of the anion upon the negatively charged particles of water.

When the collodion flask (previously bathed in gelatin) is filled with M/512 Al_2Cl_6 and dipped into a beaker with distilled water, water diffuses at first very rapidly into the flask. The water is in this case negatively charged and attracted by the Al ion and repelled by the anion. When we add small quantities of a salt with bivalent anion, *e.g.* Na_2SO_4, to the Al_2Cl_6 solution the attraction of the Al_2Cl_6 solution for water is diminished more rapidly than when we add a salt with a

monovalent anion like $NaNO_3$ or $Mg(NO_3)_2$ (Fig. 15). The Mg ion attracts negatively charged water more powerfully than the $NaNO_3$ and hence the depressing effect of $Mg(NO_3)_2$ is smaller than the depressing effect of $NaNO_3$. Fig. 16 shows that the depressing effect of the addition of H_2SO_4 upon M/512 Al_2Cl_6 is greater than the addition of HNO_3, both acids acting similarly to the potassium salts with the same anion.

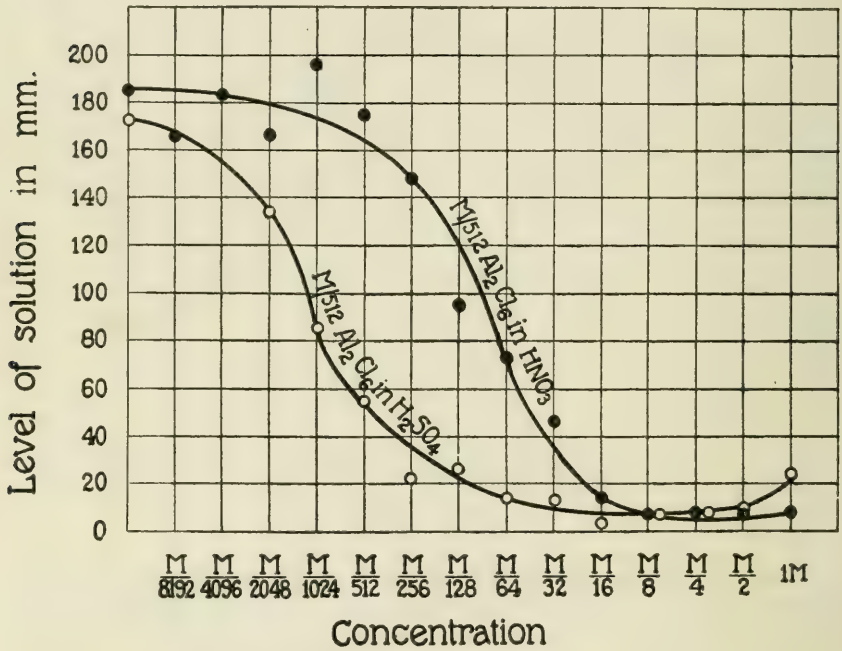

FIG. 16. Curves showing that the depressing effect of HNO_3 and H_2SO_4 is similar to the depressing effect of $NaNO_3$ and Na_2SO_4 when added to M/512 Al_2Cl_6.

All these facts support our assumption that the drop in the curves representing the influence of the concentration of the electrolyte upon the rate of diffusion of water through collodion membranes is due to the fact that beyond a certain concentration the repelling action of the ion with the same sign of charge as that of water increases more rapidly with a further increase in concentration than the attractive action of the ion with the opposite charge upon the water.

SUMMARY.

1. When a watery solution is separated from pure water by a collodion membrane, the initial rate of diffusion of water into the solution is influenced in an entirely different way by solutions of electrolytes and of non-electrolytes. Solutions of non-electrolytes, *e.g.* sugars, influence the initial rate of diffusion of water through the membrane approximately in direct proportion to their concentration, and this influence begins to show itself under the conditions of our experiments when the concentration of the sugar solution is above M/64 or M/32. We call this effect of the concentration of the solute on the initial rate of diffusion of water into the solution the gas pressure effect.

2. Solutions of electrolytes show the gas pressure effect upon the initial rate of diffusion also, but it commences at a somewhat higher concentration than M/64; namely, at M/16 or more (according to the nature of the electrolyte).

3. Solutions of electrolytes of a lower concentration than M/16 or M/8 have a specific influence on the initial rate of diffusion of water through a collodion membrane from pure solvent into solution which is not found in the case of the solutions of non-electrolytes and which is due to the fact that the particles of water diffuse in this case through the membrane in an electrified condition, the sign of the charge depending upon the nature of the electrolyte in solution, according to two rules given in a preceding paper.[1]

4. In these lower concentrations the curves representing the influence of the concentration of the electrolyte on the initial rate of diffusion of water into the solution rise at first steeply with an increase in the concentration, until a maximum is reached at a concentration of M/256 or above. A further increase in concentration causes a drop in the curve and this drop increases with a further increase of concentration until that concentration of the solute is reached in which the gas pressure effect begins to prevail; *i.e.*, above M/16. Within a range of concentrations between M/256 and M/16 or more (according to the nature of the electrolyte) we notice the reverse of what we should expect on the basis of van't Hoff's law; namely, that the attraction of a solution of an electrolyte for water diminishes with an increase in concentration.

5. We wish to make no definite assumption concerning the origin of the electrification of water and concerning the mechanism whereby ions influence the rate of diffusion of water particles through collodion membranes from pure solvent to solution. It will facilitate, however, the presentation of our results if it be permitted to present them in terms of attraction and repulsion of the charged particles of water by the ions. With this reservation we may say that in the lowest concentrations attraction of the electrified water particles by the ions with the opposite charge prevails over the repulsion of the electrified water particles by the ions with the same sign of charge as that of the water; while beyond a certain critical concentration the repelling action of the ion with the same sign of charge as that of the water particles upon the latter increases more rapidly with increasing concentration of the solute than the attractive action of the ion with the opposite charge.

6. It is shown that negative osmosis, *i.e.* the diminution of the volume of the solution of acids and of alkalies when separated by collodion membranes from pure water, occurs in the same range of concentrations in which the drop in the curves of neutral salts occurs, and that it is due to the same cause; namely, the repulsion of the electrified particles of water by the ion with the same sign of charge as that of the water. This conclusion is supported by the fact that negative osmosis becomes pronounced when the ion with the same sign of charge as that of the electrified particles of water carries more than one charge.

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HYDROGEN ION CONCENTRATION OF CULTURES OF PNEUMOCOCCI OF THE DIFFERENT TYPES IN CARBOHYDRATE MEDIA.

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Knowledge of the physiological activities and immunological characters of bacteria serves not merely the purposes of systematic classification, but contributes to a fuller understanding of the problems of infectious disease. The correlation of these apparently independent characters with pathogenicity and with the occurrence and distribution of recognizable types under a wide variety of environmental conditions is essential to the proper interpretation of the phenomena of infection.

Of the physiological characters of bacteria the fermentation of certain carbohydrates by some, and the inability of other closely related organisms to utilize the same substances, are relatively fixed characteristics of sufficient constancy to warrant their use as a basis for differentiation. In fact, within a given species these biochemical reactions are of considerable value in distinguishing type relationships. The application of this principle in the classification of the typhoid-dysentery group affords an illustration of the value of the biochemical method in determining the existence of certain varieties of these organisms and their relation to enteric infections. On the other hand, it has been possible in the case of pneumococcus to acquire knowledge of the occurrence of specific types and their relation to disease, chiefly by a study of the immunological characters of these organisms, since their biochemical activities are apparently possessed in common by all.

The measure of acid production by bacteria has been the determination of the amount of titrable acidity produced in a medium containing a known concentration of carbohydrate. This method is now recognized as inadequate. Recently the colorimetric determination

of the hydrogen ion concentration has been adapted to bacteriological requirements and affords an additional method for measuring this phase of bacterial metabolism. Moreover, in addition to the selective fermentation of certain substances by bacteria, the maximum acidity that can be tolerated by a given organism is apparently a biologic constant. Accordingly, the determination of the hydrogen ion concentration at which bacterial growth ceases has been utilized as a differential method. In the case of a culture growing in a medium containing an excess of fermentable substance this limiting reaction is spoken of as the final hydrogen ion concentration. This phenomenon has been utilized by Clark (1) and Clark and Lubs (2) in the differentiation of the colon-*aerogenes* group, and by Ayers (3), Ayers, Johnson, and Davis (4), and Avery and Cullen (5) in the recognition of differences between hemolytic streptococci of human and bovine origin.

The present paper presents the results, obtained under the experimental conditions defined, of a study of the influence of the concentration of dextrose on the final hydrogen ion concentration of broth cultures of pneumococcus, the rate of acid production, the optimum and limiting hydrogen ion concentration for initial growth of pneumococcus, the final hydrogen ion concentration of cultures of pneumococcus in carbohydrate media, and a comparison of these physiological functions with the specificity of the immunological type characters of these organisms. In addition, some information has been acquired concerning the factors governing growth of pneumococcus when reinoculated into filtrates of cultures of the same and different types.

EXPERIMENTAL.

Source of Material.—Thirty-nine strains of pneumococcus comprising representatives of the various immunological types were studied. In the majority of instances these cultures were isolated directly from the blood or sputum of patients suffering from lobar pneumonia. Some of the strains were tested immediately upon isolation, others after years of cultivation on artificial media. Most of the strains were pathogenic for white mice and had been passed through these animals to enhance virulence. Cultures used for inoculation of the test medium were grown for 18 hours at 37°C. in plain meat infusion broth of pH

7.8. The serological methods used for the determination of types of pneumococcus were the same as those already described (6).

Determination of Hydrogen Ion Concentration.—The colorimetric method of determining hydrogen ion concentration was chosen because of its convenience and simplicity. The principle and details of this method have been so thoroughly reviewed that it is unnecessary to repeat them here (7). In the present work the two principal sources of error, color of the medium and turbidity of the culture, have been avoided by first diluting the medium and then compensating for the color by Walpole's comparator method of superimposing the color of the medium upon that of the indicator.

The hydrogen ion concentrations are expressed in the customary manner as pH values; that is, the negative exponent to the base 10 of the normality.

Duplicate tubes containing 5 cc. of the medium or culture were diluted to 15 cc. with redistilled water, four drops of indicator solution were added to one tube, and the tube was compared in a comparator block with a standard solution containing exactly the same amount of indicator. The standard solutions of known hydrogen ion concentration were prepared for the range pH 8.6 to 5 from standard phosphates by Sörensen's technique, and for the range pH 5.8 to 4.6 from acetic acid-sodium acetate mixtures by Walpole's directions. The accuracy of these standard solutions was verified by the hydrogen electrode.

The indicators used were:

	per cent
pH 8.6–8.0, *o*-cresol sulfonephthalein	0.04
pH 8.0–6.8, phenolsulfonephthalein (phenol red)	0.04
pH 6.8–5.8, bromocresolsulfonephthalein, saturated water solution.	
pH 5.8–4.6, methyl red	0.04

Sterilization of the Medium Containing the Test Substance.—In studying fermentative activity of bacteria the manner in which the medium containing the test substance is sterilized is of first importance. If a sugar, such as sucrose, is added to the slightly alkaline broth and sterilized in an Arnold sterilizer or boiled for a long time, the sugar will be hydrolyzed. It seemed best, therefore, to sterilize by boiling a concentrated solution of the substance in water. Enough of this con-

centrated solution is then added to sterile broth to the desired dilution. 10 minutes in boiling water has sufficed in most cases to effect sterilization. When the medium has been handled many times, in adjusting to different reactions, further sterilization has been considered necessary. Additional sterilization is indicated in the protocol.

Influence of Sugar Concentration upon the Final Hydrogen Ion Concentration.—In studying the sugar-fermenting property of any organism it is, of course, essential to determine the influence of varying concentrations of sugar upon the final reaction. In order to do this for pneumococcus the following experiment was carried out.

To sugar-free broth of known hydrogen ion concentration sterile 20 per cent dextrose solutions (boiled 10 minutes) were added in sufficient amounts to make the desired concentration. The medium was incubated over night to test sterility, and 50 cc. portions were inoculated with 0.1 cc. of an 18 hour plain broth culture of pneumococcus. The hydrogen ion concentration of the medium was determined before the inoculation and after 24 and 48 hours incubation (Table I).

TABLE I.

Influence of the Concentration of Dextrose on the Final Hydrogen Ion Concentration of Pneumococcus Cultures.

50 cc. of sugar-free* broth, pH 7.5, inoculated with 0.1 cc. of 18 hour plain broth culture of Pneumococcus Type II (Strain F 149).

Dextrose.	Hydrogen ion concentration.	
	24 hrs.	48 hrs.
per cent	*pH*	*pH*
Broth control (uninoculated).	7.5	7.5
0	7.3	7.2
0.1	6.5	6.5
0.2	6.0	6.0
0.4	5.1	5.0
1.0	5.0	5.0
2.0	5.0	5.0
4.0	5.0	4.9

* In the preparation of sugar-free broth the meat infusion, before the addition of peptone, is fermented by *B. coli* for 18 to 24 hours.

It is evident that 0.4 per cent dextrose furnished sufficient acid to bring the medium to the limiting hydrogen ion concentration, but that excess of sugar up to at least 4 per cent has no influence upon the final reaction. 1 per cent, therefore, was chosen as sufficient for all routine fermentations. This agrees closely with a similar experiment with *Streptococcus hæmolyticus* (5).

Rate of Acid Production of Pneumococcus in 1 Per Cent Dextrose Broth.—It was to be expected that the rate of acid production would be dependent upon the size of the inoculum, but it was desirable to compare the rate curve in dextrose broth with that in plain broth. Moreover, it was necessary to determine the time required for the attainment of the final hydrogen ion concentration. In determining the rate a massive inoculum was used to bring the experiment within 1 day (Table II).

TABLE II.

Rate of Acid Production by Pneumococcus in Dextrose Broth.

100 cc. of sugar-free broth plus dextrose to 1 per cent, inoculated with 0.5 cc. of an 18 hour plain broth culture of pneumococcus, and incubated at 37°C.

Pneumococcus.	1 per cent dextrose broth. Initial hydrogen ion concentration.	Hydrogen ion concentration.					
		1½ hrs.	4 hrs.	6 hrs.	8 hrs.	9 hrs.	27 hrs.
	pH	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*
Type I	7.75	7.7	7.3	6.5	5.6	5.3	5.0
" II	7.9	7.9	7.5	6.8	5.7	5.3	4.8

No change in reaction of the dextrose broth occurred during the period of initial lag, then acid was produced at a rapid and constant rate until the final reaction was reached—in this case a pH 4.8 to 5.

With the usual inoculum employed in these experiments, 0.1 cc. of an 18 hour culture to 50 cc. of broth, the final hydrogen ion concentration is attained within 24 hours. An occasional culture will show a further increase in acidity of not more than 0.1 pH after 2 to 7 days incubation, but such changes are within the limits of experimental error. Table III furnishes an illustration of this fact. In a few instances in this experiment 18 hours was not sufficient time for the

attainment of the final reaction, but no apparent changes took place after 30 hours. As a routine, therefore, the cultures were read after 24 or 48 hours incubation at 37°C.

TABLE III.

Relation of the Type of Pneumococcus to the Final Hydrogen Ion Concentration in Sugar Broth.

75 cc. portions of 1 per cent sugar broth having an initial reaction of pH 7.8 were inoculated with 0.1 cc. each of an 18 hour plain broth culture of pneumococcus of different types.

Pneumococcus.		Hydrogen ion concentration.					
		Dextrose.			Lactose.		
Type.	Strain.	18 hrs.	30 hrs.	5 days.	18 hrs.	30 hrs.	5 days.
		pH	*pH*	*pH*	*pH*	*pH*	*pH*
I	F 55	5.1	5.1	5.1	5.1	5.1	5.1
I	146	5.0	5.1	5.0	5.0	5.0	5.0
II	46	4.9	5.0	5.0	5.0	5.0	5.0
II	F 149	5.2		5.1	5.1	5.1	5.1
IIa	J	5.8	5.0	5.0	5.3	4.9+	5.0
IIb	W	5.1	5.2	5.2	5.3	5.1	5.1
III	A 66	5.1	5.05	5.05	5.1	5.0	5.1
III	F 104	5.0	5.0	5.0	5.1	5.0	5.1
IV	L F	5.0	5.0	4.9	5.1	5.2	5.1
IV	L A	5.0	5.2	5.2	5.1	5.2	5.2

Relation of the Type of Pneumococcus to the Final Hydrogen Ion Concentration of Cultures in Sugar Broth.—In order to determine whether the differences in antigenic properties of the specific types of pneumococcus had any influence on the final reaction in sugar medium, two strains of each of the four types, as well as strains representing subgroups of Type II were inoculated into broth containing 1 per cent dextrose or lactose. It is evident from Table III that the final pH of all strains of pneumococcus was between pH 5.2 and 4.9, with the majority between pH 5.0 and 5.2. The differential characters as determined by immunological reactions were not evident from the final hydrogen ion concentrations. It is of significance that the final hydrogen ion concentration of cultures of pneumococcus is exactly the same as the final reaction of pathogenic hemolytic streptococci of

human type. This is also brought out in Table IV where the results of all the strains used in these and succeeding experiments are summarized by types. This table represents 54 determinations on 39 strains. Duplicate determinations in individual experiments are not included in this table.

TABLE IV.

Final Hydrogen Ion Concentration of Pneumococcus in Dextrose Broth. Summary of 54 Determinations on 39 Strains.*

Type I.		Type II		Type III.		Type IV.	
Strain.	Hydrogen ion concentration.	Strain.	Hydrogen ion concentration.	Strain.	Hydrogen ion concentration.	Strain.	Hydrogen ion concentration.
	pH		*pH*		*pH*		*pH*
I	5.2	II	5.0	A 66	5.0	L F	5.0
I	5.0	II	4.9	A 66	5.1	L A	5.2
I	5.1	F 149	5.1	F 104	5.0	F 194	5.1
F 55	5.1	F 149	5.1	F 104	4.8	X 47	5.2
E 22	5.1	F 149	5.1	D 40	5.0	E 190	5.0
F 152	5.0	F 149	5.1	D EH	4.9	E 190	4.8
D 6	5.0	F 149	5.0	U	5.1	E 117	5.0
D 46	5.0	F 149	5.0	E 84	5.0	E 157	5.0
D 46	4.8	F 149	5.1	C 28	5.0	E 121	5.2
D 46	5.0	F 149	5.3	E 111	4.9	D 107	5.0
16,887	5.0	D 39	5.0	G	5.0	Sch.	4.9
16,867	5.0	IIa J	5.0	E 127	5.0		
F 169	5.0	IIb W	5.0	A	4.9		
		IIc A	5.0				
		IIc 50	5.0				
		II atypical, F 150	5.0				
		II " F 154	5.1				
Total 13		17		13		11	
Average.....	5.0		5.0		4.9		5.0

* Repetition of strains in the above table represents observations made in different experiments.

It is evident from the preceding experiment that with dextrose and lactose there is no type difference in acid production. A number of other carbohydrates fermentable by pneumococcus have been tested.[1]

[1] The substances used were either Kahlbaum's reagents or were those prepared by Mr. E. P. Clark of The Rockefeller Institute for Medical Research.

The media were prepared by placing solutions or suspensions of the substance in boiling water for 10 minutes, and adding the required amount to 75 cc. of media. The media were then heated in the Arnold sterilizer once for 20 minutes, and incubated to test sterility. They were inoculated with 0.1 cc. each of 18 hour plain broth cultures of pneumococcus representing the four types. Samples were removed for pH determinations after 48 hours and 7 days. Since 7 day readings are practically identical with 48 hour readings, only the latter are given (Table V).

TABLE V.

Final Hydrogen Ion Concentration of Pneumococcus in Various Carbohydrate Media.

75 cc. of sugar-free broth containing 1 per cent carbohydrate, initial pH 7.5, were inoculated with 0.1 cc. of an 18 hour plain broth culture and incubated 48 hours at 37°C.

Test substance.	Uninoculated control.	Pneumococcus type.			
		I (Strain F 152).	II (Strain F 149).	III (Strain F 104).	IV (Strain E 190).
	pH	*pH*	*pH*	*pH*	*pH*
Maltose	7.5	5.0	5.0	4.9	5.0
Saccharose	7.5	5.0	5.1	5.0	5.0
Lactose	7.5	5.0	5.0	5.0	4.9
Galactose	7.3	5.1	5.1	5.1	4.8
Raffinose	7.5	5.0	5.1	5.1	5.1
Dextrose	7.5	5.1	5.1	4.8	4.8
Inulin	7.5	5.1		5.1	5.1

From the results presented in Table V it is evident that in media containing sufficient fermentable carbohydrate, growth of pneumococcus continues until a final hydrogen ion concentration of about pH 5 is reached. Apparently this acidity is sufficient in itself to stop growth and the organisms die in the products of their own metabolism. Cultures of pneumococci with all the carbohydrates which were fermentable under the conditions used, namely maltose, saccharose, lactose, galactose, raffinose, dextrose, and inulin, gave identical results in the rate of reaction change and final hydrogen ion concentration (pH 5) attained. Further, the different immunological types of pneumococcus, in a limited number of strains studied, behave alike in fermenting these carbohydrates.

Optimum Hydrogen Ion Concentration for Growth of Pneumococcus.—Work previously reported from this laboratory (8) showed that the optimum hydrogen ion concentration for the growth of pneumococcus was about pH 7.8, and that the hydrogen ion concentrations within which growth could be initiated were between pH 7.0 and 8.3. It was desirable to determine whether the optimum pH in sugar broth was the same as that in plain broth and also to determine for dextrose broth the upper and lower hydrogen ion concentrations beyond which initial growth does not occur.

50 cc. portions of plain broth containing 0.2 per cent phosphate and the same broth containing 1 per cent dextrose were adjusted to the desired reactions by addition of either sodium hydroxide or hydrochloric acid. The media were placed in boiling water for 10 minutes, and incubated for 48 hours to test sterility. They were then inoculated with 0.25 cc. of an 18 hour culture of Pneumococcus Type I (Strain F 169). After 6 hours, estimation of growth and pH determinations were made, and a portion was autoclaved for determination of turbidity by the nephelometer method (Kober instrument). Essentially the same procedure was used as that described by Dernby and Avery (8), except that it seemed unnecessary to estimate the number of organisms per cubic centimeter, since it is in reality a function of the size of the inoculum. The readings on the nephelometer were expressed, therefore, as percentages of the heaviest growth (Table VI).

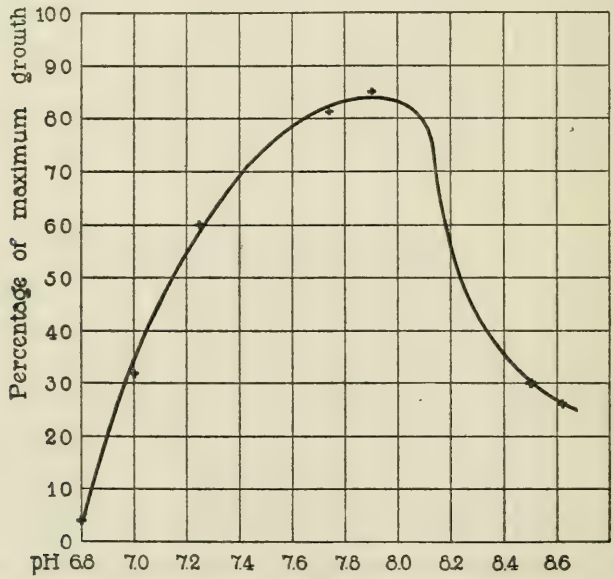
This method is entirely satisfactory with plain broth, and the results are in agreement with those of Dernby and Avery (8); it is beset with some difficulty, however, in the case of sugar broth. With plain broth the experiment may be continued to the maximum growth possible, but in sugar broth the readings must be made before acid precipitation takes place. Slight variations in reading and in initial lag are therefore emphasized. However, the curve constructed from the results was, with one exception, smooth (Text-fig. 1). The experiment was repeated with the same general result but with discrepancies in individual tubes. Another experiment was then planned, in which in addition to the turbidity method, pH determinations were made at regular intervals in order to give an idea of the time required to reach a given pH.

TABLE VI.

Optimum Hydrogen Ion Concentration for Growth of Pneumococcus.

50 cc. portions of plain broth and 1 per cent dextrose broth were adjusted to the indicated hydrogen ion concentration and inoculated with 0.25 cc. of Pneumococcus Type I (Strain F 169). Readings were made after 6 hours at 37°C.

Initial hydrogen ion concentration of media.	Plain broth.		Dextrose broth.	
	Turbidity.	Hydrogen ion concentration.	Turbidity.	Hydrogen ion concentration.
pH	*per cent*	*pH*	*per cent*	*pH*
8.4	23	8.1	80	7.5
8.3	70	7.5		
8.0	73	7.4	100	7.2
7.8	65	7.1	37	7.5
7.5	39	7.1	39	7.1
7.3		6.9	16	7.1
7.0		6.9		6.9
6.5				6.5
6.0				6.0
5.5				5.5



TEXT-FIG. 1. Effect of initial hydrogen ion concentration on growth of pneumococcus in dextrose broth.

100 cc. portions of plain broth containing 1 per cent dextrose were adjusted to the desired pH, and 25 cc. removed for controls. After 24 hours at 37°C. as a sterility test, the 75 cc. were inoculated with 0.5 cc. of a 12 hour broth culture of Pneumococcus Type I–1 (this heavy dose was given in order to complete the experiment within 1 day). At intervals 5 cc. samples were removed for pH determinations, and when these readings indicated active growth 5 cc. samples were autoclaved for nephelometer readings. Macroscopic estimations of growth were also made to a +++ scale. The results are given in Table VII and in Text-fig. 2.

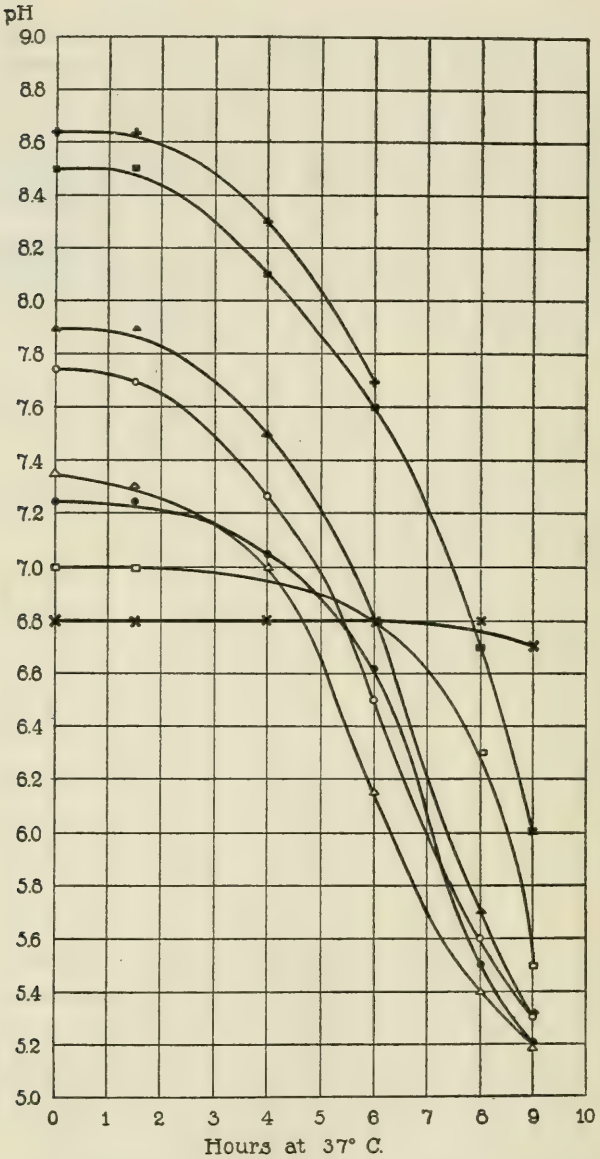
TABLE VII.

Optimum Hydrogen Ion Concentration for Growth of Pneumococcus in Dextrose Broth.

75 cc. of plain broth containing 1 per cent dextrose were inoculated with 0.5 cc. of a 12 hour broth culture of Pneumococcus Type I–1.

Hydrogen ion concentration.						Growth.		Turbidity. 6 hrs.
Control.	1½ hrs.	4 hrs.	6 hrs.	8 hrs.	9 hrs.	1 hr.	6 hrs.	
pH	*pH*	*pH*	*pH*	*pH*	*pH*			*per cent*
6.8	6.8	6.8	6.8	6.8	6.7	−	±	4.2
7.0	7.0	7.0	6.8	6.3	5.5	−	+	32.0
7.25	7.25	7.05	6.6+	5.5	5.2	+	+++	60.0
7.35	7.3	7.0	6.15	5.4	5.2	+	+++	
7.75	7.7	7.3	6.5	5.6	5.3	+	+++	82.0
7.9	7.9	7.5	6.8	5.7	5.3	−	+++	85.0
8.5	8.5	8.1	7.6	6.7	6.0	−	+±−	30.0
8.6+	8.6+	8.3	7.7			−	+	26.0

It is evident from these experiments that the optimum hydrogen ion concentration for growth of pneumococcus in dextrose broth was about pH 7.8; that is, the same as for plain broth (Text-fig. 1). Cultures grow luxuriantly between pH 7.2 and 8.6. The upper limit for growth in sugar medium is somewhat higher than in plain medium. Although fermentation in sugar broth starts at a pH of 7.8 and proceeds until a pH of 5 is reached, it is not possible to initiate growth below pH 7.0. The curve of the rate of acid production for this series has been also constructed (Text-fig. 2). At pH 7.75 to 7.3 the initial lag is decreased, but in all the tests from pH 7.2 to 8.6 after the initial



TEXT-FIG. 2. Effect of initial hydrogen ion concentration upon the rate of reaction change due to growth of pneumococcus in dextrose broth.

lag luxuriant growth occurs at about the same rate. This experiment emphasizes the fact that the term limiting hydrogen ion concentration must be carefully defined and that the final hydrogen ion concentration may be entirely different from the hydrogen ion concentration limits within which growth may be initiated.

Limiting Initial Hydrogen Ion Concentration.—It is brought out in the preceding experiments that the ordinary inoculation of pneumococcus fails to initiate growth if the hydrogen ion concentration of the medium is greater than pH 7.0, but that with growth well started at a pH above 7.0, acid production proceeds in sugar media to a final pH of 5.0. It would seem possible then that either the bacteria rapidly adapt themselves to the changing and hitherto unfavorable reaction, or that the medium itself is rendered more suitable for growth.

If the explanation lies in rapid adaptation of the organism one would expect that bacteria removed from a sugar broth at a reaction of pH 6.0, for example, could grow in fresh broth of the same reaction. An attempt to test this point was carried out as follows:

Dextrose broth was inoculated with 0.5 cc. of an 18 hour culture of Pneumococcus Type I. The change in reaction was followed, and at pH of 7.1, 6.5, 5.5, and 5.0 fresh samples of dextrose broth of varying hydrogen ion concentrations were inoculated with the actively growing culture. This broth had previously been adjusted to the desired reaction, tubed in 5 cc. portions, and incubated to test sterility. The heavy inoculation of 0.1 cc. of culture per 5 cc. of medium was used, and in addition several tubes were inoculated with the massive dose of 0.5 cc. of culture to 5 cc. of medium. The 13 hour culture (pH 5) was still viable as evidenced by the occurrence of growth when inoculated into fresh broth of pH 7.8. Only traces of disintegration of the cells were visible under the microscope. This experiment is recorded in Table VIII.

Study of Table VIII shows that it is impossible to initiate growth in media having a more acid reaction than pH 6.8 regardless of the fact that the inoculum may have been removed from an actively growing culture at ranges of acidity from pH 7.1 to 5.0. For instance, if the organisms are alive and growing at a pH 5.5, and a seeding is removed at this point and implanted in medium with a reaction of pH 6.5, no growth occurs.

TABLE VIII.

Failure of Actively Growing Cultures at pH below 6.8 to Initiate Growth in Fresh Broth of pH below 6.8.

5 cc. of dextrose broth of the indicated reaction were incubated for 24 hours at 37°C. after inoculation with cultures which had developed an acid reaction.

Culture (Type I).		1 per cent dextrose broth.	Inoculation per 5 cc.			
			0.1 cc.		0.5 cc.	
Age.	Hydrogen ion concentration.		Growth.	Final hydrogen ion concentration.	Growth.	Final hydrogen ion concentration.
hrs.	*pH*	*pH*		*pH*		*pH*
6½	7.1	7.8	++++	4.9		
8	6.5		++++	4.8		
9	5.5		++++	4.9		
13	5.0		++++	4.9		
6½	7.1	7.0	−	7.0−		
8	6.5		−	6.8+	++++	4.9
9	5.5		++++	5.0	++++	5.0
13	5.0		++++			
6½	7.1	6.8	−	6.8		
8	6.5		++++	4.9	++++	4.9
9	5.5		−	6.5	++++	4.9
13	5.0		−	6.8		
6½	7.1	6.5	−	6.5		
8	6.5		−	6.3	−	6.1
9	5.5		−	6.35	−	6.1
13	5.0		−	6.4		
6½	7.1	6.0	−	6.0		
8	6.5		−	6.0	−	5.9
9	5.5		−	5.95	−	5.8
13	5.0		−		−	5.8

Growth of Pneumococcus in Filtrates of Dextrose Broth Cultures of Pneumococcus.—An attempt was next made to determine whether dextrose broth in which pneumococcus has grown to the final hydrogen ion concentration will sustain growth if the organisms are removed by Berkefeld filtration and the reaction of the filtrate is readjusted to ranges of acidity from pH 7.8 to 6.0.

To salt-free broth 0.2 per cent sodium phosphate and 1 per cent dextrose were added, the medium was adjusted to a pH 7.8, and inoculated with Type I pneumococcus. After 24 hours incubation the culture was filtered through a Berkefeld filter. Samples of the filtrate, the pH of which was 5.2, were adjusted with sodium hydroxide to pH 6.0, 6.5, 6.8, 7.0, and 8.0; 5 cc. of filtrate required 3.3 cc. of 0.05 N sodium hydroxide to bring it to a pH 8.0. The filtrate at each pH was divided into 5 cc. portions, one of which was used as a test for sterility. Tubes containing 5 cc. of filtrate were inoculated in duplicate with 0.1 cc. of 18 hour cultures of Types I and II. The results are given in Table IX.

TABLE IX.

Growth of Pneumococcus in Filtrates of Dextrose Broth Cultures of Pneumococcus.

Dextrose broth pH 7.8. After 24 hours the culture was filtered through a Berkefeld filter. The pH of the filtrate was 5.2.

Filtrate from Type I (Strain 183) adjusted to.	48 hrs. after inoculation with.			
	Type I (Strain 183).		Type II (Strain D 39).	
	Growth.	Hydrogen ion concentration.	Growth.	Hydrogen ion concentration.
pH		*pH*		*pH*
8.0	++++	5.3		
7.0	++++	5.2	++++	5.1
6.8	++++	5.2	++++	5.2
6.5	−	6.5	−	6.5
6.0	−	6.0	−	6.0

It is evident that filtrates of dextrose broth in which pneumococcus has grown and the reaction of which has been readjusted with sodium hydroxide will not allow growth to be initiated if the readjusted pH is below 6.8.

This experiment indicates that the medium is not specifically exhausted of the substances necessary for growth, nor is there a formation of specific inhibiting bodies. This has been confirmed by growing two other strains of different types of pneumococcus in dextrose broth. After a week the cultures became sterile, the organisms were removed by centrifugation, the supernatant fluids were adjusted to

a pH 7.7, and portions of each fluid were reinoculated from fresh cultures of both organisms. Growth occurred in all portions (Table X).

TABLE X.

Dextrose Broth in Which Pneumococcus Has Grown Shows No Specific Exhaustion of Fermentable Substances and No Specific Inhibiting Substances.

1 per cent dextrose broth, pH 7.7, after 7 days incubation, broth sterile, pH 5.1. The supernatant fluid after centrifugation was adjusted with sodium hydroxide to pH 7.7, reinoculated, and incubated for 48 hours.

Supernatant fluid of broth cultures of pneumococcus.	Adjusted hydrogen ion concentration of supernatant fluid.	Reinoculation of supernatant fluid with pneumococcus.	
		Type I.	Type II.
	pH	*pH*	*pH*
Type I	7.7	5.1	5.1
" II	7.7	5.1	5.1

Exhaustion of Fermentable Substances in Plain Broth.—Since growth cannot be started in either plain broth or in dextrose broth if the hydrogen ion concentration is appreciably greater than pH 7.0 to 6.8, it is evident that this degree of acidity is unfavorable for the initiation of growth. However, pneumococcus in dextrose broth of pH above 7.0 grows to a pH of 5.0, while in plain broth of the same initial pH, growth ceases at about 7.0. Is this cessation of growth a result of the attaining of that reaction, or is it due to exhaustion of fermentable substances? To test this, plain broth cultures of pneumococcus which had developed maximum acidity, were filtered through a Berkefeld filter and the filtrate was divided into several portions and reinoculated with pneumococcus. As seen in Table XI the filtrate, both at pH 7.0 and when readjusted to its initial pH 7.5, shows no growth; on the other hand, the addition of dextrose, whether to the unadjusted filtrate, or to filtrate the reaction of which has been restored to the initial pH, is sufficient to cause abundant growth. It is evident then that the cessation of growth in plain broth when the culture has reached about pH 7.0 is due, partially at least, to exhaustion of fermentable substances. The acid produced in itself does not inhibit growth. This conclusion is emphasized by Table I in which it is shown that 0.1 per cent dextrose broth reaches a pH 6.5, 0.2 per cent dextrose broth a pH 6.0, and 0.4 per cent dextrose broth a pH 5.0.

TABLE XI.

Exhaustion of Fermentable Substances in Plain Broth by Pneumococcus.

100 cc. of plain broth, initial pH 7.5, were inoculated with Type I (Strain D 46), at 37°C. for 24 hours, and filtered through a Berkefeld filter. The filtrate, pH 7.0, was reinoculated and incubated 48 hours.

Filtrate of plain broth culture of Pneumococcus Type I.	Hydrogen ion concentration of filtrate.	Filtrate reinoculated with pneumococcus.			
		Type I.		Type II.	
		Growth.	Hydrogen ion concentration.	Growth.	Hydrogen ion concentration.
	pH		*pH*		*pH*
Unchanged	7.0	−	7.0	−	7.0
Dextrose added to 1 per cent	7.0	+++	5.0	+++	5.1
Adjusted with NaOH	7.5	−	7.5	−	7.5
" " NaOH + 1 per cent dextrose	7.5	+++	5.0	+++	5.0

DISCUSSION.

In this paper are presented facts thus far acquired in a study of acid production by pneumococcus when grown in the presence of fermentable substances. The strains of pneumococcus chosen are representative of the different serological types and in the majority of instances have been isolated from patients having lobar pneumonia. Some of these strains were freshly isolated, others have been under cultivation on artificial media for years. During this time their virulence has been maintained by animal passage and they have lost none of their biologic specificity. It is evident also from the protocols given that these conditions of preservation and animal passage have not affected the biochemical functions concerned in acid production. Moreover, it is apparent, for the limited number of strains studied at least, that no difference in the degree of acidity produced by the specific types of pneumococcus could be determined in media containing the test substances. In measuring the acidity produced by growth of pneumococcus use has been made of the colorimetric method for determining the hydrogen ion concentration. The limit of acid tolerance of pneumococcus in sugar-containing media, representing the final hydrogen ion concentration, is remarkably constant for all the strains studied.

The point of maximum acidity at which growth ceases has been defined as the final hydrogen ion concentration and has been found for all types of pneumococcus in carbohydrate media to be about pH 5. This final reaction is affected by the concentration of fermentable sugar in the medium up to 0.4 per cent. In the presence of this amount of dextrose, for instance, sufficient acid is produced during growth to bring the medium to the final hydrogen ion concentration of pH 5, but excess of sugar up to at least 4 per cent has no further influence on the final reaction. The rate of acid production, while a function of the size of the inoculum and of the optimum reaction of the medium, is, as previously shown by Chesney (9) and Cullen and Chesney (10), after the period of initial lag, rapid and constant until the final hydrogen ion concentration is reached.

In media containing 1 per cent maltose, saccharose, lactose, galactose, raffinose, dextrose, or inulin, strains of pneumococcus representing the four specific types produced acid to a final hydrogen ion concentration of pH 5. These results are in agreement with those of a previous study of 48 strains in which it was observed that these test substances are fermented by pneumococcus regardless of type differences.

Dernby and Avery have previously shown that the optimum hydrogen ion concentration for growth of pneumococcus in plain broth is pH 7.8. In the course of the present experiments these results have been confirmed and the observations extended to determine the optimum reaction for growth in media containing sugar. The initial reaction of all the media tested shows the optimum to be pH 7.8. The limits of hydrogen ion concentration within which growth can be initiated, however, were found to cover a somewhat wider range in carbohydrate-containing media (pH 8.3 to 6.8) than in similar media to which these substances are not added (pH 8.1 to 7.0). However, it is apparently impossible to initiate growth in a medium containing sugar if the initial reaction is more acid than pH 6.8, even though the organisms used for seeding are transplanted immediately from an actively growing culture at ranges of acidity varying from pH 7.1 to 5.

It has been found that in bacteria-free filtrates of plain broth cultures of pneumococcus, even when the filtrate is adjusted to the opti-

mum reaction, pneumococcus cannot be made to grow again unless small amounts of sugar (dextrose) are added. On the other hand, filtrates of dextrose broth cultures of pneumococcus, under the optimum conditions of reaction, apparently contained sufficient unutilized sugar to allow growth to occur on subsequent reinoculation. The ability of pneumococcus to grow, then, when reinoculated into the filtrate of a broth culture of pneumococcus of the same or different type, after the reaction of the filtrate has been readjusted to the optimum hydrogen ion concentration, appears to be dependent in part at least upon the presence of a residuum of fermentable substance left unmetabolized by previous growth. It is probable, however, that the exhaustion of fermentable carbohydrate from culture media is only one of many factors involved in the complex phenomenon of growth inhibition.

SUMMARY.

1. The optimum hydrogen ion concentration for growth of pneumococcus is pH 7.8.

2. In broth cultures growth of pneumococcus continues until a final hydrogen ion concentration of about pH 5.0 is reached, if sufficient fermentable carbohydrate (above 0.4 per cent) is present. Apparently this acidity is sufficient in itself to stop growth.

3. If less carbohydrate is present in the medium growth ceases at a lower hydrogen ion concentration, apparently because of exhaustion of carbohydrate. If no carbohydrate is present save that extracted from the meat of which the broth is made (plain broth medium), growth initiated at pH 7.8 (optimum reaction) ceases at about pH 7.0.

4. If bacteria-free filtrates of plain broth cultures in which growth has ceased are readjusted to pH 7.8 and reinoculated with pneumococcus, no growth occurs unless carbohydrate is added. However, if bacteria-free filtrates of dextrose broth cultures in which growth has ceased (pH 5) are readjusted to pH 7.8 and reinoculated with pneumococcus growth occurs.

5. Cultures of pneumococcus with all the carbohydrates which were fermentable under the conditions used, namely maltose, saccharose, lactose, galactose, raffinose, dextrose, and inulin, gave identical results in the rate of reaction change, and final hydrogen ion concentration (pH 5.0) attained.

6. The different immunological types of pneumococcus, for the limited number of strains studied, behaved alike in fermenting the carbohydrates mentioned above.

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DIRECT DETERMINATION OF NON-AMINO NITROGEN IN THE PRODUCTS OF PROTEIN HYDROLYSIS.

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In the previous method used by Van Slyke[1] for analyzing proteins according to the chemical groups characteristic of the different amino-acids, the non-amino nitrogen was determined indirectly by calculating the difference between the total nitrogen of the amino-acids, estimated by Kjeldahl and the amino nitrogen, estimated by the Van Slyke method. As results thus calculated are subject to the added errors of the two determinations, an attempt has been made to devise a method by which the non-amino nitrogen could be estimated directly from a single determination.

Principle of the Method.

The procedure is exactly the same as that used in the former method for protein analysis[1,2] until the hexone bases have been separated from the remaining amino-acids. At this point we turned our attention to the filtrate from the bases.

Since the bases are precipitated with phosphotungstic acid, the filtrate contains the excess of this acid as well as that used in the washings. It was found necessary to remove all traces of the phosphotungstic acid before proceeding, as its presence interferes with the reduction of nitrites by the zinc-copper couple which occurs later in the procedure.

In the estimation of the total nitrogen of the filtrate and the amino nitrogen, however, it is important that the determination be performed before removal of the phosphotungstic acid, because the amyl alcohol-ether mixture which we use for extracting the phos-

[1] Van Slyke, D. D., *J. Biol. Chem.*, 1911–12, x, 15.

[2] Van Slyke, D. D., *J. Biol. Chem.*, 1915, xxii, 281.

photungstic acid[2] also extracts some of the amino nitrogen, as is shown by Table V. Peculiarly, it does not extract any of the non-amino nitrogen.

After the removal of the phosphotungstic acid with amyl alcohol and ether, the solution is ready to use for the determination of the non-amino nitrogen. This was accomplished in three steps.

1. The Removal of Primary Amino Nitrogen by Reaction with Nitrous Acid.—$RNH_2COOH+HNO_2 = N_2+H_2O+ROHCOOH$. In order to reduce the amount of inorganic nitrogen introduced to a minimum small amounts of sodium nitrite are used for the production of nitrous acid, and the reaction is accelerated by heat instead of by a large excess of reagents.

At first small amounts of glacial acetic acid were used for the production of nitrous acid. On testing out the method on a solution of proline, the results were found somewhat high. That this error was not due to an incomplete removal of the amino nitrogen is shown by Table I. That it was not due to an incomplete reduction of the nitrite is shown by Table II. A further test was made by varying the amount of proline used for testing out the method. It was found that the increase in nitrogen over that calculated was directly proportional to the amount of proline used. This is shown in Table III. It seems that there is a fixation of some of the nitrite nitrogen presumably attached to the proline as nitroso-nitrogen, $RNH+HNO_2=RN-NO+H_2O$. In order to break up such compounds hydrochloric acid was substituted for acetic, and theoretical results were obtained.

2. The Removal of Excess Nitrous Acid.—The nitrogen of the nitrous acid which reacts with NH_2 groups escapes as N_2 gas, but some of the excess nitrous acid necessary for the completion of the reaction remains. This must be quantitatively removed before the residual non-amino organic nitrogen can be determined by Kjeldahl. Several methods were tried, but the only one which gave quite satisfactory results was reduction to ammonia by the zinc-copper couple. The technique used by Scales[3] was adapted to our purpose. The reduction is carried out in a boiling solution made alka-

[3] Scales, F. M., *J. Biol. Chem.*, 1916, xxvii, 327.

line with magnesium oxide, so that the ammonia can be distilled off as formed.

3. The Determination of the Residual, Non-Amino Nitrogen.—This was done by the ordinary Kjeldahl method.

Details of Procedure in Protein Analysis.

The filtrate from the hexone bases,[1,2] representing 2 or 3 gm. of protein, is concentrated according to the original method of Van Slyke[1] to a volume of 150 cc. Portions of this solution in our experiments have been used for determination of the total nitrogen and amino nitrogen as described in Van Slyke's original paper. A portion of the remainder was freed of phosphotungstic acid and used for determination of non-amino nitrogen, as described below.

Total Nitrogen in the Filtrate from the Bases.—10 cc. portions were taken for Kjeldahl, using 10 gm. of potassium sulfate, 0.2 gm. of copper sulfate, and 20 cc. of concentrated sulfuric acid.

Amino Nitrogen in the Filtrate from the Bases.—2 cc. portions were used for the amino determination, using the micro-apparatus in the usual manner with a 5 minute reaction period.

Direct Determination of Non-Amino Nitrogen in the Filtrate from the Bases.—Of the 150 cc. of filtrate 100 cc. are measured into a separatory funnel of 500 cc. capacity. 5 cc. of concentrated hydrochloric acid and 75 cc. of a mixture of equal parts of amyl alcohol and ether are added. The funnel is shaken 1 or 2 minutes, and the aqueous layer separated. The extraction is repeated with three more successive portions of amyl alcohol-ether, using 50, 25, and 25 cc. for the successive extractions. Finally the combined amyl alcohol-ether extracts are shaken out once with water to remove traces of water solution which might have been carried along. This washing is extracted once with fresh amyl alcohol-ether, and combined with the main solution. Solutions thus treated were found free of phosphotungstic acid when tested with barium hydroxide. The solution is concentrated in vacuum to a volume of about 50 cc. and washed into a 100 cc. volumetric flask, thus making the solution to the original volume of filtrate taken. The results of this treatment on the non-amino nitrogen are shown in Table IV. In pro-

teins where the non-amino nitrogen is very low, the volume could be made smaller. In case a heavy white precipitate appears on concentration, and does not go into solution on diluting and shaking, the solution is filtered after diluting to the required volume.

20 cc. portions of the solution are used for the non-amino nitrogen determination. Each portion is pipetted into a small Kjeldahl flask to which 1.2 cc. of 30 per cent sodium nitrite and 5 cc. of concentrated hydrochloric acid (sp. gr. 1.2) are added. The flasks are suspended in a boiling water bath for 45 minutes. The solution is then neutralized to alizarin with 10 per cent sodium hydroxide.

While this reaction is taking place, the zinc-copper couples may be prepared as described by Scales.[3,4] The neutralized solution from which the nitrite nitrogen is to be removed is washed quantitatively on to the couple, and then about 0.75 gm. of magnesium oxide is added, the sides of the flask are washed down, and the volume is made up to 200 or 250 cc. The solution is now boiled gently for 45 minutes in a hood with a good draught. The reaction is completed and the ammonia removed in this time, as is shown by Table II.

After the completion of the reaction, the solution is immediately washed from the couple into a clean dry 500 cc. Kjeldahl flask, washing the zinc four times with distilled water. The zinc is covered with water until ready for the next determination. The solution is treated with 10 gm. of potassium sulfate, 0.2 gm. of copper

[4] Sheet zinc of about 0.5 or 0.25 mm. thickness is cut into strips 2 cm. by 8 cm. These strips are coiled by rolling loosely around a pencil, or better, with a pair of large forceps. 80 gm. of these coils are dropped into a previously weighed 500 cc. Kjeldahl flask, so that the weight of the flask plus coils is known. When the zinc is used for the first time, it is covered with an approximately 1 per cent sulfuric acid solution for 3 or 4 minutes, then a 1 per cent sulfuric acid solution for 3 or 4 minutes, until the surface is clean. In case the zinc has already been used, it is washed once with water, then covered with 1 per cent sulfuric acid until clean. The acid solution is drained off, and the zinc is covered with an acid copper sulfate solution prepared by dissolving 10 gm. of $CuSO_4.5H_2O$ in 2 liters of distilled water and then adding 6 cc. of concentrated sulfuric acid. This is allowed to stand for at least 8 minutes, until the zinc has become well coated. It is then poured off and the couple washed once with water.

sulfate, and 20 cc. of concentrated sulfuric acid, boiled down, and digested for 1 hour after it clears.

Purity of Reagents.—Every reagent used must be carefully tested by blank analyses, and the correction ascertained must be applied to each determination. The phosphotungstic acid is purified with ether and water.[5] The zinc seems to be the most uncertain of all the reagents, and the most likely to cause error. When used for the reduction of nitrites in protein digests, the zinc very soon deteriorates. For this reason it is necessary to test the couples frequently with a known proline solution, or else to renew the zinc frequently. The zinc may be used for three to six determinations, keeping the weight constant by adding more coils if necessary, but it is safest to test out the coils after the third determination. New coils should always be tested with a known proline solution before using.

EXPERIMENTAL.

To test the time required for the decomposition of amino nitrogen with nitrous acid, a 1 per cent solution of glycocoll was used. In the first three determinations, Table I, 10 cc. of a 1 per cent solution were

TABLE I.

Time Required for the Completion of Decomposition of the Amino Nitrogen in a Glycocoll Solution.

Acid used.	Time of reaction.	NH_2-N found.	NH_2-N used.	Decomposition.
	min.	*mg.*	*mg.*	*per cent*
Acetic	15	0.319	18.6	98.28
"	30	0.024	18.6	99.87
"	45	0.000	18.6	100.00
Hydrochloric	30	0.412	39.2	98.95
"	45	0.000	39.2	100.00

used, representing 18.6 mg. of amino nitrogen; in the last two, 20 cc. were used, representing 39.2 mg. of amino nitrogen, which is the maximum that is likely to occur in the amount of solution used in an analysis of protein. The glycocoll solution was measured into a

[5] Winterstein, E., *Z. physiol. Chem.*, 1901–02, xxxiv, 153.

small Kjeldahl flask, and 1.2 cc. of a 30 per cent solution of sodium nitrite were added, with, in the first three determinations, 0.3 cc. of glacial acetic acid, in the last two 5 cc. of concentrated hydrochloric acid. The flasks were immersed in a boiling water bath. At stated intervals flasks were removed from the bath, neutralized with sodium hydroxide, made to volume, and amino determinations were made on 2 cc. portions. The results are given in Table I.

To test the time required for the reduction of nitrite nitrogen and its complete removal as ammonia, an experiment was performed using the amount of nitrite required in the method described for the direct determination of non-amino nitrogen, and proceeding with the method as described. During the reduction with zinc-copper couple the ammonia was distilled into standard acid and titrated. The results are given in Table II.

TABLE II.

Time Required for Reduction of Nitrite Nitrogen with Zinc-Copper Couple.

Time of reaction.	Nitrogen reduced.	
min.	*cc. N/14*	*mg. N*
45	33.31	33.31
	33.72	33.72
Next 15.	0.05	0.05
	0.00	0.00

The method for the direct determination of non-amino nitrogen was tested on proline and oxyproline. 20 cc. of a 0.7 per cent solution of the copper salt of proline were used, representing the maximum amount of non-amino nitrogen present in a determination as described for proteins. For the oxyproline determinations 20 cc. of a 1 per cent solution were used. In part of the determinations 0.3 cc. of glacial acetic acid was tried in place of the 5 cc. of concentrated hydrochloric acid. The results of the test and also of the use of different acids are given in Table III.

A test was next made to find out the effect of treatment with amyl alcohol-ether mixture on the determination of non-amino

TABLE III.

Effect of Determination in Presence of Glacial Acetic and Hydrochloric Acids on Results of Non-Amino Nitrogen Determination.

Substance used.	Amount used.	Acid used.	Non-amino nitrogen.		
			Calculated.	Found.	Error.
	mg.		*mg.*	*mg.*	*mg.*
Proline	70	Acetic.	5.40	6.40	+1.0
"	140	"	10.80	12.80	+2.0
"	140	Hydrochloric.	10.80	10.73	−0.07
Oxyproline	100	Acetic.	1.27	1.52	+0.25
"	200	"	2.54	3.05	+0.51
"	250	Hydrochloric.	2.54	2.60	+0.06

nitrogen. A 100 cc. portion of the above mentioned proline solution was treated exactly as described above for the filtrate from the bases in hydrolyzed proteins. 10 cc. portions of the solution were used for the determinations. In determining the calculated non-amino nitrogen of the proline it was necessary to perform both a Kjeldahl and an amino determination, and then subtract the amino nitrogen ("Amino nitrogen before treatment"). This slight amount of amino nitrogen is due to traces of amino-acids present as impurities in the proline used. The latter was made from hydrolyzed protein by the ester method and purified as the copper salt, and it is difficult to free it completely from traces of other amino-acids. The results of this test are given in Table IV.

TABLE IV.

Effect of Treatment with Amyl Alcohol-Ether Mixture on the Determination of Non-Amino Nitrogen in Proline.

Total nitrogen. before treatment.	Amino nitrogen before treatment.	Non-amino nitrogen.		
		Calculated.	Found.	Loss.
mg.	*mg.*	*mg.*	*mg.*	*per cent*
5.69	0.23	5.45	5.36	2.0
5.69	0.23	5.45	5.26	2.5

Casein was put through the procedure as described for proteins, and the total and amino nitrogen of the filtrate from the bases determined both before and after treatment with amyl alcohol-ether mixture. The results of the treatment are given in Table V.

TABLE V.

Effect of Treatment with Amyl Alcohol-Ether Mixture in Removing Amino Nitrogen, but not Non-Amino Nitrogen, from the Filtrate from the Bases. Hydrolyzed Casein.

	Before treatment.	After treatment.	Loss.
	mg.	*mg.*	*per cent*
Total nitrogen	194.0	165.6	14.7
Amino "	165.0	137.0	17.0
Non-amino "			
Indirect*	29.0	28.6	1.4
Direct		28.56	1.2

* Difference between total and amino nitrogen.

Analyses of Proteins.

Casein.—12 gm. of casein were hydrolyzed with 120 cc. of 20 per cent hydrochloric acid for 46 hours, and the solution brought to 250 cc. Of this, 50 cc. portions, containing, according to Kjeldahl determinations, 0.2878 gm. of nitrogen each were used. The following determinations were made on the filtrate from the bases.

Total nitrogen, using 10 cc. portions.
Amino " " 2 " "
Non-amino " " 20 " "

The results are given in Table VI.

Gelatin.—10 gm. of gelatin were hydrolyzed with 200 cc. of 20 per cent hydrochloric acid for 40 hours, and the solution brought to 250 cc. Of this, 75 cc. portions containing, according to Kjeldahl determinations, 0.4420 gm. of nitrogen each were used. The following determinations were made on the filtrate from the bases.

Total nitrogen, using 10 cc. portions.
Amino " " 2 " "
Non-amino " " 20 " "

The results are given in Table VI.

TABLE VI.

Results of Analyses of Protein.

	Casein.							Gelatin.						
	Total nitrogen.	Amino nitrogen.		Non-amino nitrogen.				Total nitrogen.	Amino nitrogen.		Non-amino nitrogen.			
				Indirect.		Direct.					Indirect.		Direct.	
	gm.	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*
Uncorrected I	0.1940	0.1650		0.0290		0.0286		0.3226	0.2459		0.0767		0.0760	
Uncorrected II	0.1900	0.1625		0.0275		0.0269		0.3258	0.2472		0.0786		0.0782	
Corrected for solublity of bases I		0.1598	55.60	0.0241	8.38	0.0237	8.24		0.2407	54.40	0.0718	16.22	0.0711	16.07
Corrected for solublity of bases II		0.1573	54.70	0.0226	7.87	0.0220	7.65		0.2420	54.75	0.0737	16.66	0.0733	16.56
Average of corrected results		0.1586	55.20	0.0233	8.12	0.0229	7.97		0.2414	54.60	0.0728	16.46	0.0722	16.33

SUMMARY.

In the mono-amino-acid fraction of the products of protein hydrolysis, as analyzed by the method of Van Slyke, the non-amino nitrogen, comprising the nitrogen of proline, oxyproline, and one-half that of the tryptophane, can be determined directly by a single Kjeldahl determination. The amino nitrogen is removed by warming with sodium nitrite and hydrochloric acid, the excess of nitrous acid is reduced with a zinc-copper couple by Scales' method, and the non-amino nitrogen in the residue determined by Kjeldahl.

The results by this method agreed closely, in analyses of casein and gelatin, with the results obtained indirectly by Van Slyke's original procedure, in which the non-amino nitrogen is calculated as the difference between total nitrogen and amino nitrogen.

It appears, therefore, that the original method, although indirect, contains no inherent errors, and that when due precautions in regard to calibrated apparatus and standardized solutions are observed, results of practically the same degree of accuracy can be obtained by the original, somewhat simpler indirect method, as by the present direct one.

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THE CONTROL OF ACIDOSIS IN THE TREATMENT OF DIABETES.

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INTRODUCTION.

During the past five years marked advances have been made in the treatment of diabetes, due largely to the introduction by Allen of the "fasting method." In the practical application of this method of treatment the patient is fasted until glycosuria disappears; and subsequent to the initial fast, fasting is employed to such an extent as is necessary to aid in keeping the patient free from glycosuria. In other words, the most important and significant sign or symptom by which the therapeutic regime is regulated is the presence or absence of sugar in the urine. Allen, however, has emphasized the fact that in diabetes a disturbance of the entire metabolic mechanism of the body is present, and that the presence or absence of glycosuria at any given time is but a crude test of the state of that function, though practically it is the best test available. In the latest publication,[1] stress is laid on the importance of the state of the blood sugar, and attention is also drawn to the importance of acidosis.

The questions of the nature of acidosis and the relationship between changes in alkaline reserve and clinical symptoms have been discussed in previous papers from this Hospital (Van Slyke and Cullen;[2] Stillman, Van Slyke, Cullen and Fitz;[3] Fitz,[4] 1917; Palmer and Van Slyke[5]). Suffice it to say that although other factors, including, per-

1. Allen, F. M., Stillman, E., and Fitz, R.: New York, Monograph of the Rockefeller Institute, 1919, No. 11.

2. Van Slyke, D. D., and Cullen, G. E.: J. Biol. Chem. **30**: 289, 347, 389, 401, 405 (June) 1917.

3. Stillman, E., Van Slyke, D. D., Cullen, G. E., and Fitz, R.: J. Biol. Chem. **30**: 405 (June) 1917.

4. Fitz, R.: Proc. Assn. Am. Phys. **32**: 155, 1917; **30**: 389, 405 (June) 1917.

5. Palmer, W. W., and Van Slyke, D. D.: J. Biol. Chem. **32**: 499 (Dec.) 1917.

haps, unknown toxins, can also cause diabetic collapse (Fitz[4]), as a rule, the severity of the clinical symptoms parallels the fall in plasma bicarbonate; moreover, the plasma bicarbonate begins to fall before the symptoms appear.

The introduction of more exact methods for the study of acidosis, in particular, methods for determining blood plasma bicarbonate, and the opportunity for combined clinical and laboratory studies on a group of patients, selected chiefly for the severity of their diabetes, have resulted in the gradual development in this clinic of systematic procedures, first, for ascertaining the individual reactions of the patient in regard not only to this carbohydrate tolerance, but also his tendency toward acidosis, and second, for guiding the treatment by the metabolic reactions that have been ascertained. The present paper is an outline of these procedures.

Laboratory Control.

The minimum laboratory control with which satisfactory results may be considered possible consists of qualitative glucose and acetoacetic acid tests on the daily urine samples, with determinations of plasma bicarbonate when the urine shows a strong test for acetoacetic acid, or when any of the clinical signs are unfavorable. The symptoms of incipient acidosis are extremely variably; they may include indefinite malaise, headache, slight nausea, neuralgic pains, or almost any abnormal symptom. Consequently, the slightest unfavorable clinical sign at any stage of treatment should be taken as an indication for determining the plasma bicarbonate.

It is desirable, also, especially for patients whose condition is uncertain or who are being put on a new regimen, to determine quantitatively the blood sugar and the daily excretion of sugar and acetone bodies in order to follow the progressive effect of the treatment. We have usually determined the blood sugar whenever blood was drawn for bicarbonate estimation.[6]

6. The methods used in this clinic have been the following: Blood plasma bicarbonate: CO_2 capacity method, Van Slyke and Cullen[2] (the titration method of Van Slyke, Stillman and Cullen[7] was not available at the time these observations were made). Blood sugar: Lewis and Benedict method, as modified by

Outline of Routine.

For the most expeditious creation and maintenance of an aglycosuric and aketogenic state, the routine evolved in applying the principles of the fasting treatment in this hospital may be outlined as follows:

1. A preliminary observation diet.
2. The creation of the aglycosuric and aketogenic state by either continuous or intermittent fast.
3. Estimation of the individual carbohydrate tolerance.
4. Determination of the maintenance diet.
5. Final observation period.

The significance, according to our experience, of acidosis in each stage of the treatment and its consideration in modifying the treatment are indicated in the following pages.

1. The Preliminary Observation Diet.—On admission to the hospital patients not suffering from acidosis have been placed on the following observation diet in order to ascertain their behavior under standardized conditions (see Table 4).

Acidosis of the moderately severe or severe type (plasma bicarbonate CO_2 below 40 volumes per cent.) has been considered the one contraindication to placing the patient on the observation diet and maintaining him on it for the usual length of time. Such patients have been put on fasting directly on admission to the hospital as requiring immediate and active treatment (Table 5).

Myers and Bailey.[8] Urinary sugar: qualitative, Benedict;[9] quantitative, Benedict (1911). Acetone bodies in urine: qualitative, the well known ferric chlorid test of Gerhardt, described in all laboratory manuals; quantitative Van Slyke.[10] Acetone bodies in blood: quantitative, Van Slyke and Fitz.[11] Titrable acids in urine: Folin.[12] Ammonia in urine: Van Slyke and Cullen.[13]

7. Van Slyke, D. D., Stillman, E., and Cullen, G. E.: J. Biol. Chem. **38**: 167 (May) 1919.
8. Myers, V. C., and Bailey, C. V.: J. Biol. Chem. **24**: 147 (Feb.) 1916.
9. Benedict, S. R.: J. Biol. Chem. **5**: 485, 1908; **9**: 57, 1911.
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11. Van Slyke, D. D., and Fitz, R.: J. Biol. Chem. **32**: 495 (Dec.) 1917; **37**: 551 (April) 1919.
12. Folin, O.: Am. J. Physiol. **9**: 265, 1903.
13. Van Slyke, D. D., and Cullen, G. E.: J. Biol. Chem. **19**: 211, 1914; **24**: 117 (Feb.) 1916.

2. Creation of the Aglycosuric State by Continuous or Intermittent Fast.—After the period of the observation diet, the patient is placed on a continuous fast (Tables 4, 5 and 6). This is continued until the patient is rendered aglycosuric unless acidosis develops. By the continuous fast is meant absolute abstinence from all foods containing any demonstrable amount of carbohydrate, protein or fat, continued without interruption until the patient has been made sugar free for twenty-four hours. In our series of cases this has required from two to eleven days. Fluids, in the form of clear soup, coffee and water, are taken daily in a total amount of at least from 1,500 to 2,000 c.c.

In occasional instances (one out of sixteen in our cases which were, of course, selected for their severity) continuous fasting has resulted in the development of dangerous acidosis. Consequently, patients on continuous fasts have been watched carefully for this development. The clinical symptoms of incipient acidosis have been found to vary

TABLE 1.

Observation Diet.

Protein, Gm.	Carbohydrate, Gm.*	Fat, Gm.
1.5 per kilogram	10–25	Sufficient to bring total calories to 35 per kilogram body weight

* Carbohydrate in the form of green vegetables only, from 10 to 25 gm. according to apparent severity of the case.

so extremely that any local or general malaise has come to be looked on with suspicion. The only reliable tests have been found to be those of the laboratory. It seems well to emphasize here that we have come to believe that during the fasts accurate daily determinations of the plasma bicarbonate should be performed to obviate a certain percentage of fatalities.

In the cases developing or retaining acidosis on the continuous fast (Table 7), as shown by plasma bicarbonate much below normal, continuous fast has been changed to the intermittent fast. Its object is the same as that of the continuous fast—namely, the creation of an aglycosuric state—and its substitution has been made solely when acidosis has resulted from the more expeditious continuous method.

By the intermittent fast is meant an absolute fast, for periods of time depending on the reaction of the patient, from all foods containing any demonstrable amount of carbohydrate, protein or fat, the fasting periods being interrupted by intervals of diets low in caloric value (see Table 7 and accompanying description).

The manner in which the continuous and intermittent fasting treatments have been applied has been governed by the state and behavior of the bicarbonate reserve of the patient's blood.

(a) In cases with no acidosis or only mild acidosis, as determined before the institution of fasting, a moderate fall in the plasma bicarbonate has been permitted before the continuous fast was abandoned for the intermittent.

(b) If the existing acidosis was moderately severe (plasma bicarbonate CO_2 of from 30 to 40 volume per cent.) even a drop of two or three volume per cent. in bicarbonate, especially if the fall was progressive, was sufficient to cause interruption of the fast.

(c) In cases in which the bicarbonate determination showed a severe acidosis (plasma bicarbonate CO_2 below 30 volume per cent.) active treatment was instituted as follows:

Sodium bicarbonate: 3 gm. per hour, dissolved in cold water, until the bicarbonate of the blood became normal.

Fluids: Forced to the limit, but not to the extent to cause nausea; it has been attempted to give about 5,000 c.c. of fluid during the twenty-four hours.

Coffee: Clear, very strong coffee has been given to stimulate the renal action of patients suffering from severe acidosis. As much as from 1,200 to 1,500 c.c. of coffee has been given during the day.

Whisky: No beneficial effect on the acidosis has been obtained from giving alcohol, and it has apparently nauseated in some instances; therefore, the use of whisky as a therapeutic measure for acidosis has been discontinued in this hospital.

Catharsis: Active catharsis by calomel and salts, together with high colon irrigations until the washings are returned without fecal matter, has been instituted. We have no exact data as to the part which this treatment has had in ameliorating the acidosis, but clinically the effects have seemed to be beneficial.

Diet: Continuous fasting has been applied directly on admission. If from six to twelve hours' fasting, together with the above treatment, did not cause an increase in the plasma bicarbonate, light feeding was begun. It was thought theoretically correct that such feeding should be principally protein, as carbohydrate in such cases is not burnt, and could only add to the severity of the diabetes, while fat leads to the formation of ketone bodies and consequent direct increase in acidosis. Therefore, from 600 to 800 calories have been given in the form of eggs and lean meat (see Table 7).

In the treatment of acidosis we have found that nausea is the condition above all others to be avoided, because if it becomes uncontrollable, successful treatment of the acidosis becomes nearly impossible. Nausea may be produced by any one of the above mentioned measures—bicarbonate therapy, the forcing of fluids, catharsis, the institution of fasting or the institution of feeding. Experience has taught us that close clinical observation and frequent laboratory tests can, in the great majority of cases, prevent the development of nausea, if at the first sign of any intensification of if the offending factor in the treatment, whether it be forced fluids, bicarbonate, fasting or feeding, be discontinued until the symptoms of nausea disappear.

The statements bearing on the degree of acidosis and the laboratory indications for changing from continuous to intermittent fasting are summarized in Table 2.

Estimation of the Individual Carbohydrate Tolerance on a Diet of Green Vegetables Alone.—After the patient has been made aglycosuric by the fasting method and has remained aglycosuric for twenty-four hours, he has been placed on a graduated carbohydrate tolerance test. This test has been performed by giving a diet comprised of green vegetables alone, starting the test with green vegetables containing 10 gm. of carbohydrate and increasing the amount of carbohydrate 10 gm. each day, until glycosuria results (Tables 4, 5, 6 and 7). Occasionally a trace of sugar has been found to occur in the urine during the first days of the test. This not infrequently has been found to clear up without fasting, and the patients have frequently shown an ability to metabolize many times the amount of carbohydrate that the first sugar reaction seemed to indicate was the limit of their tolerance. Therefore, on the first appearance of glycosuria we have continued the same amount of carbohydrate for another twenty-four hours; if, at the end of this time, there was cessation of the sugar excretion, the test was continued, the carbohydrate food being increased daily until glycosuria appeared

on two successive days. This point we have considered as indicating the carbohydrate tolerance for the individual under observation. Fasting was then again instituted until the patient had become sugar free for twenty-four hours. The determination of the maintenance diet was then begun.

The carbohydrate tolerance test establishes a basis for comparison with later tests, whereby the progress of the patient can be determined, and also affords a basis for the initial maintenance diet as described below.

TABLE 2.

Degree of Acidosis and Laboratory Indications for Changing from Continuous to Intermittent Fasting.

Degree of Acidosis	CO_2 Combining with 100 C.c. of Blood Plasma, C.c.	Acid Excretion* $\frac{N/10\ NH_3 + Acid}{Kg.\ Body\ Weight}$ C.c.	Drop of CO_2 Permitted before Interrupting Fast, C.c.
No acidosis	Over 53	0 to 27	To 45
Mild acidosis	53 to 40	27 to 65	Drop of 10 to 5
Moderately severe acidosis	40 to 30	27 to 100	Drop of 3 to 2
Severe acidosis	Below 30	Over 100	Fast interrupted after 6 to 12 hours unless CO_2 rises with treatment outlined above

* Concerning the interpretation of the relationship of the urinary acid excretion figure, $\frac{N/10\ NH_3 + \text{titratable acid}}{\text{Kg. body weight}}$, to severity of acidosis, the reader is referred to the discussion in paper X of the Studies of Acidosis (Van Slyke, 1918). The ordinary correspondence between these figures and the degree of acidosis is indicated by the table.

In cases in which although acidosis has been benefited by fasting, but has not been entirely eradicated, the carbohydrate test diet has usually raised the blood bicarbonate to a full normal. The ketonuria, also, which has usually accompanied subnormal blood bicarbonate, has been diminished or entirely stopped. The green vegetable diet may be considered almost a specific for mild acidosis. (Note its effect in Tables 4, 5 and 6 in raising plasma bicarbonate and abolishing the ferric chlorid reaction for acetoacetic acid in the urine.)

Determination of the Maintenance Diet.—By the maintenance diet is meant a mixed diet, which is as near a normal diet as can be tolerated without glyco-

suria and with fully normal plasma bicarbonate. After having made the patient aglycosuric and having determined the carbohydrate tolerance, it has been our custom to place him on a trial maintenance diet (Table 3).

TABLE 3.

The Trial Maintenance Diet.

Per 24 Hours.		
Carbohydrate	Protein	Fat
One tenth the amount tolerated on the carbohydrate tolerance test	1.5 gm. per kg. body weight	Sufficient to bring the total caloric value of the diet to 35 calories per kg. of body weight

If the diet was well tolerated and the patient appeared to need more, an increase was made in the constituents of the diet which appeared to be most needed. Care was taken that only one of the three constituents, either the fat, the protein or the carbohydrate, was increased at a time and in a gradual systematic manner, so that should glycosuria or ketonuria result the offending food could be recognized and reduced.

Urinary tests for glucose and ketones have been made daily, while the plasma bicarbonate has been determined at least once a week, and as often after as the condition and symptoms of the patient rendered advisable (see p. 470).

When either glycosuria, definite ketonuria, or significant fall in plasma bicarbonte occurred on the maintenance diet, the patient has been put on the so-called minimum maintenance diet, which was made up exactly as in the maintenance diet, except that the fat was cut down so that the patient received only twenty-five calories per kilogram body weight instead of thirty-five. Prolonged feeding (for from four to twelve weeks) of a diet of such low caloric content has frequently resulted in increased food tolerance, so that the patient could finally take the maintenance or even more than the maintenance diet. When it has been found that it was impossible to create such a tolerance, patients have been discharged on the lower diet.

If at the end of two weeks on the finally chosen maintenance diet, there has been no indication of glycosuria, no significant increase in

TABLE 4.

Dietary Record of Case 2234, Group 1.

Date, 1916	Day of Observation	Body Weight, Kg.	Diet				Dextrose		Evidence of Acidosis						NaHCO$_3$ by Mouth
									Urine					Blood: CO$_2$ Combined with 100 C.c. Plasma, C.c.	
			C. H., Gm.	Protein, Gm.	Fat, Gm.	Total Diet, Calories	Urine, Gm.	Blood, per Cent.	FeCl$_3$ Reaction	N/10 NH$_3$ C.c.	N/10 Acid C.c.	N/10 NH$_3$ + N/10 Acid C.c.	N/10 NH$_3$ + Acid / Kg. Body Weight, C.c.		
Jan. 25	1	50.4	10	60.0	130	1,490	3.25	....	Moderate						
26	2	50.8	60	60.0	130	1,490	Trace	....	Heavy						
27	3	50.2	60	60.0	130	1,490	2.55	....	Moderate						
28	4	49.8	Fast				Trace	0.17	Moderate	255	218	473	9.5	61.0	
29	5	49.8	Fast				Negative	....	Moderate						
30	6	49.6	Fast				Negative	....	Moderate						
31	7	48.4	Fast				Negative	....	Moderate						
Feb. 1	8	47.8	10	4.0	0.3	60	Negative	....	Moderate						
2	9	48.0	20	7.5	1.0	120	Negative	0.15	Moderate	200	187	387	8.1	63.1	
3	10	48.0	30	10.5	1.9	180	Negative	0.14	Moderate						
4	11	48.2	40	14.0	2.2	240	Trace	0.11	Moderate	142	204	346	7.2	65.5	
5	12	48.4	50	17.5	2.4	300	Trace	....	Trace						
6	13	48.6	60	20.0	3.1	355	Negative	....	Negative						
7	14	48.0	70	23.0	4.6	425	Negative	0.10	Negative	54	83	137	2.85	72.3	
8	15	48.0	80	24.0	4.8	470	Negative	....	Negative						
9	16	48.2	90	31.0	5.1	545	Negative	0.12	Negative	43	67	110	2.3	73.9	
10	17	48.4	100	31.5	5.0	585	Negative	....	Negative						
11	18	48.6	110	34.5	5.5	645	Negative	0.09	Negative	43	42	85	1.75	68.8	
12	19	48.8	120	37.0	6.9	705	Negative	....	Negative						
13	20	48.2	130	39.0	7.0	755	Negative	....	Negative						
14	21	48.6	140	39.5	6.3	795	Negative	0.18	Negative	39	70	109	2.25	70.4	
15	22	48.8	150	43.5	7.0	860	Negative	....	Negative						
16	23	49.2	115	31.0	3.3	615	Negative	0.11	Negative	33	71	104	2.1	71.1	

TABLE 5.

Dietary Record of Case 2686, Group 2.

Date, 1916	Day of Observation	Body Weight, Kg.	Diet				Dextrose		Evidence of Acidosis						NaHCO$_3$ by Mouth
									24 Hour Urine					Blood: CO$_2$ Combined with 100 C.c. Plasma, C.c.	
			C. H., Gm.	Protein, Gm.	Fat, Gm.	Total Diet, Calories	Urine, Gm.	Blood, per Cent.	$FeCl_3$ Reaction	N/10 NH_3 C.c.	N/10 Acid C.c.	N/10 NH_3 + N/10 Acid C.c.	N/10 NH_3 + Acid / Kg. Body Weight, C.c.		
Feb. 22	1	....		Fast*			23.0*	0.27	Heavy	*	*	*	*	12.2	45
23	2	27.8		Fast			42.0	0.38	Heavy	2,025	835	2,850	102	26.8	50
24	3	26.0		Fast			24.5	0.32	Heavy	1,508	598	2,106	81	38.3	30
25	4	25.4		Fast			21.0	0.28	Heavy	1,710	533	2,243	88	40.4	30
26	5	25.0		Fast			12.0	0.26	Heavy	1,495	286	1,781	70	46.0	20
27	6	25.2		Fast			12.0	....	Heavy	1,735	298	2,033	81	....	15
28	7	25.4		Fast			5.5	0.25	Heavy	1,772	220	1,992	78	49.6	
29	8	25.2		Fast			Trace	....	Moderate						
Mar. 1	9	25.2		Fast			Negative	0.21	Moderate	1,417	152	1,569	63	48.7	
2	10	25.0	10	3.9	0.4	60	Trace	....	Trace						
3	11	25.6	20	8.6	1.0	125	Slight	0.20	Trace	830	62	892	35	43.0	
4	12	26.6	20	8.6	1.0	125	Trace	....	Negative						
5	13	28.0	25	10.0	1.0	150	Trace	....	Trace						
6	14	30.0	25	10.0	1.0	150	Trace	0.22	Negative	798	47	845	28	55.0	
7	15	31.4	25	10.0	1.0	150	Trace	....	Negative						
8	16	31.8	30	11.5	1.3	180	Trace	0.26	Negative	550	42	592	19		
9	17	31.8	35	13.0	1.5	210	Trace	....	Negative						
10	18	31.6	8.8†	3.3	0.4	50	Trace	0.23	Negative	318	169	487	15	57.6	
11	19	30.4		Fast			Negative	....	Negative						
12	20	30.0	5	1.5	0.2	25	Negative	....	Negative						
13	21	29.8	10	3.8	0.4	60	Negative	....	Negative	365	57	422	14		

14	22	29.8	20	7.7	0.8	120	Negative	....	Negative						
15	23	29.4	20	8.6	0.9	125	Negative	0.12	Negative	244	48	292	12	53.6	
16	24	28.0	30	11.3	1.4	180	Negative	....	Negative						
17	25	28.4	40	14.5	1.8	240	Negative	....	Negative						
18	26	28.4	50	18.1	2.2	300	Negative	....	Negative						
19	27	28.6	60	21.3	2.5	355	Negative	....	Negative						
20	28	27.6	70	24.3	2.7	410	Negative	....	Negative						
21	29	26.4	80	27.3	3.2	470	Negative	0.15	Negative	....	...	....	...	55.6	
22	30	26.0	90	29.9	4.5	530	Negative	....	Negative						
23	31	26.0	100	33.9	6.1	605	Trace	....	Negative						
24	32	25.2	100	33.9	6.1	605	Trace	....	Negative						
25	33	2.50	110	36.0	6.8	660	Trace	....	Negative						
26	34	25.0		Fast			Negative	....	Negative						
27	35	25.2	15	40.0	61.5	795	Negative	....	Negative						
28	36	25.6	15	40.0	83.4	1,000	Negative	0.10	.....	...	...	....	...	52.2	

* Observation day of 11 hours. † Fast two meals.

TABLE 6.

Dietary Record of Case 2480, Group 3.

Date, 1916	Day of Observation	Body Weight, Kg.	Diet				Dextrose		Evidence of Acidosis						NaHCO₃ by Mouth
									24 Hour Urine					Blood: CO_2 Combined with 100 C.c. Plasma C.c.	
			C. H., Gm.	Protein, Gm.	Fat, Gm.	Total Diet, Calories	Urine, Gm.	Blood, per Cent.	$FeCl_3$ Reaction.	N/10 NH_3 C.c.	N/10 Acid C.c.	N/10 NH_3 + N/10 Acid C.c.	N/10 NH_3 + Acid / Kg. Body Weight, C.c.		
Oct. 15	1	48.4	3.5	26.8	92.7	985	Heavy	0.42	Heavy						
16	2	47.6	5.0	100.0	166.0	2,000	Heavy	....	Heavy	602	—	—	—	44.5	
17	3	47.6		Fast			Heavy	....	Heavy	926	—	—	—		
18	4	47.9		Fast			Heavy	....	Heavy	757	—	—	—		
19	5	47.4		Fast			Heavy	....	Heavy	798	—	—	—		
20	6	45.5		Fast			Heavy	....	Moderate						
21	7	45.5		Fast			Heavy	0.38	Moderate	...	...	...	....	53.2	
22	8	45.4		Fast			Heavy	....	Moderate						
23	9	45.4		Fast			Moderate	0.27	Moderate	...	...	...	....	50.4	
24	10	45.2		Fast			Slight	....	Slight						
25	11	45.4		Fast			Negative	0.38	Slight	368	151	519	11	47.8	
26	12	45.3	10	3.5	0.5	60	Negative	0.32	Slight	...	...	...	....	46.6	
27	13	46.1	20	8.0	1.0	125	Negative	0.27	Slight	298	101	399	9	47.5	
28	14	46.5	30	11.1	1.7	185	Negative	0.24	Negative	500	92	592	13	57.9	
29	15	46.9	40	15.9	2.0	250	Trace	0.40	Negative	296	82	378	8	56.5	
30	16	47.2	40	15.9	2.0	250	Negative	0.23	Negative	136	31	167	3	54.4	
31	17	47.2	50	17.8	5.8	315	Negative	....	Negative						
Nov. 1	18	47.6	60	20.0	4.6	375	Trace	0.14	Negative	...	...	...	....	53.5	
2	19	47.8	60	20.0	4.0	365	Negative	....	Negative	210	7	217	6		

3	20	47.8	70	23.0	4.6	425	Negative	0.32	Negative	...	...	...	....	57.1
4	21	47.9	80	26.3	5.7	485	Trace	....	Negative					
5	22	47.6	90	27.7	6.6	545	Slight	....	Negative					
6	23	47.6	25*	10.0	1.2	155	Slight	....	Negative					
7	24	46.8		Fast			Negative	....	Negative					
8	25	47.8	5	75.0	126.8	1,505	Negative	0.17	Negative					
9	26	47.4	0	75.0	127.5	1,495	Negative	....	Negative					
10	27	47.8	0	75.0	126.0	1,480	Trace	0.28	Negative	...	...	...	....	52.3

* Partial fast day.

TABLE 7.

Dietary Record of Case 2414 (Third Admission), Group 4.

Date, 1916	Day of Observation	Body Weight, Kg.	Diet				Dextrose		Evidence of Acidosis						NaHCO₃ by Mouth
									24 Hour Urine					Blood: CO_2 Combined with 100 C.c. Plasma, C.c.	
			C. H., Gm.	Protein, Gm.	Fat, Gm.	Total Diet, Calories	Urine, Gm.	Blood, per Cent.	$FeCl_3$ Reaction	N/10 NH_3 C.c.	N/10 Acid C.c.	N/10 NH_3 + N/10 Acid C.c.	N/10 NH_3 + Acid / Kg. Body Weight, C.c.		
Feb. 19	1	44.6		Fast			20	0.26	Heavy	....	....	....	...	26.4	
20	2	44.9	...	162.8	19.0	845	100	0.44	Heavy	4,160	1,330	5,490	122	24.2	30
21	3	44.8	...	94.0	22.8	600	87	0.47	Heavy	4,080	1,000	5,080	114	34.3	40
22	4	44.6	...	94.0	22.8	600	179	0.26	Heavy	3,885	955	4,840	108	36.0	20
23	5	44.6	...	94.0	22.8	600	103	0.26	Heavy	3,825	992	4,817	108	44.5	40
24	6	45.0	...	63.0	15.2	400	68	0.20	Heavy	2,830	706	3,536	89	47.2	40
25	7	46.0	...	63.0	15.2	400	59	0.25	Heavy	2,105	675	2,780	60	53.3	40
26	8	47.0	...	63.0	15.2	400	54	....	Heavy	....	....	....	...	65.5	40
27	9	48.4	...	31.5	7.6	200	48	....	Heavy	1,705	148	1,853	38	....	25
28	10	49.0	...	31.5	7.6	200	29	0.27	Heavy	1,900	122	2,202	41	60.9	
29	11	49.8	...	31.5	7.6	200	36	....	Moderate						
Mar. 1	12	48.0		Fast			24	0.28	Moderate	890	60	950	20	60.2	
2	13	48.0		Fast			23	....	Slight						
3	14	47.4		Fast			10	0.27	Slight	620	60	680	14	54.0	
4	15	47.2		Fast			Trace	....	Negative						
5	16	47.2		Fast			Negative	....	Negative						
6	17	47.0	10	3.8	0.8	65	Trace	0.29	Negative	396	32	428	9	64.1	
7	18	47.2	10	3.8	0.8	65	Trace	....	Negative						
8	19	47.2	15	6.5	1.1	100	Trace	0.33	Negative	350	18	308	7	54.4	
9	20	46.2	20	7.7	0.8	120	Trace	....	Negative						

10	21	46.2	25	10.0	1.0	150	Trace	0.31	Negative	263	8	271	6	64.8
11	22	46.1	30	11.5	1.3	180	Slight	....	Negative					
12	23	45.5	3.3	1.8	0.2	20	Trace	....	Negative					
13	24	44.4	...	14.9	10.6	160	Negative	0.26	Negative	257	24	281	6	61.7
14	25	44.3	...	22.3	15.9	240	Negative	.	Negative					
15	26	44.4	10	3.8	0.4	60	Negative	0.22	Negative	318	24	332	7	56.2
16	27	44.0	20	8.6	0.9	125	Negative	....	Negative					
17	28	44.2	30	11.3	1.4	180	Trace	....	Negative					
18	29	44.0	10.3	26.3	16.3	300	Trace	....	Negative					
19	30	43.0		Fast			Negative	....	Negative					
20	31	43.0	...	50.0	64.4	805	Negative	....	Negative					
21	32	43.6	..	50.0	64.4	805	Negative	0.25	Negative	.. .	....	....	...	58.3

the glycemia, and the plasma bicarbonate remains stable at a fully normal level, one may feel fairly secure that the patient in question is tolerant of the maintenance diet.

Examples of Results with Different Types of Diabetes.

Tables 4, 5, 6 and 7 exemplify the results obtained with patients belonging to the four groups previously described by the author (Stillman[14]). These groups are marked by characteristic differences in their tendencies toward acidosis which are briefly summarized as follows:

Group 1—46.8 Per Cent. of Our Cases.—No tendency to acidosis, either on fast or ordinary diet, regardless of degree of glycosuria.

Treatment: Made aglycosuric without danger by continuous fast. Can usually be made to tolerate fairly high calory diet without glycosuria or ketonuria. Easiest group to handle. Best prognosis.

Group 2—32.3 Per Cent. of Our Cases.—Received in condition of moderate or severe acidosis (plasma bicarbonate CO_2 below 40 volume per cent.), which clears up on fast.

Treatment: Made free of glycosuria and acidosis by continuous fast. A tendency toward acidosis is present, and the safely tolerated maintenance diet is usually lower than in Group 1.

Group 3—14.5 Per Cent. of Our Cases.—Tendency to slight acidosis (subnormal plasma bicarbonate) on any but most carefully chosen diet. Acidosis not increased by fasting.

Treatment: Glycosuria removed by continuous fast. Tolerated maintenance diet variable. Usually there is a tendency toward acidosis which may become acute on any improper diet.

Group 4—6.4 Per Cent. of Our Cases.—Either develop or retain severe acidosis (plasma bicarbonate CO_2 below 30 volume per cent.) when fasted to attain the aglycosuric state. This is incomparably more severe than the mild acidosis observed as a rule, when normal individuals are fasted and may rapidly become fatal unless the fall in plasma bicarbonate is checked.

Treatment: Continuous fast must be given up for intermittent fast (described below). As in Group 2, the presence of a tendency toward acidosis is demonstrated and must be considered in the future feeding and observation of the patient.

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14. Stillman, E.: Am. J. M. Sc. **151**: 505 (April) 1916.

Group 1.—Case 2234, patient aged 54 years, diabetic for five years, emaciation and clinical symptoms of moderate severity, but mild in ability to tolerate a sufficient diet for maintenance.

The table shows no tendency to acidosis during the period of preliminary observation (January 25 and 26) or during the fasting period (January 28 to 31). The subsequent carbohydrate tolerance test (February 1 to 16) shows a tolerance of at least 150 gm. of carbohydrate when given in the form of green vegetables alone. At this point the patient refused further hospital treatment, but subsequently he tolerated a maintenance diet of 40 calories per kg. body weight, including 30 gm. carbohydrate and 1.5 gm. protein per kg. body weight. (From Jan. 25 to Feb. 16, 1916.)

Group 2.—Case 2686, patient aged 13 years, diabetic one year, extreme in acid intoxication. The patient was admitted on the verge of coma, being so stuporous that it was impossible to attract his attention by any means. The combining power of the blood for CO_2 (12.2 volume per cent.) agreed with the clinical picture in indicating extreme acidosis. Therefore, the usual preliminary observation period was omitted. Fasting was immediately instituted, and sodium bicarbonate given by mouth (together with the other therapeutic measures described in the text). Within twenty-four hours the CO_2 combining power of the plasma had risen to 26.8 volume per cent., and there was a corresponding improvement in the clinical condition of the patient, consciousness having completely returned and signs of coma having disappeared. After five days fast the plasma CO_2 was nearly normal for a child 13 years of age; the patient was alert and apparently in as good condition as could be expected in a normal child after such a fast. The fast was continued for four days more, however, until the patient became aglycosuric.

The subsequent carbohydrate tolerance test showed a tolerance of 90 gm. of carbohydrate when given in the form of green vegetables alone, and a maintenance diet of 40 calories per kg. body weight including 30 gm. of carbohydrate and 2 gm. of protein per kg. was tolerated without glycosuria or acidosis.

A year and a half after admission word was received that the patient had died in coma following a break in diet. (From February 22 to March 28, 1916.)

Group 3.—Case 2480, aged 27. Diabetic four years, severe in carbohydrate intolerance and in a continuous low grade acid intoxication despite dietetic treatment. The summary shows a low grade acidosis during the preliminary observation diet (October 15 and 16) and during the subsequent fasting period (October 17 to 25). During the carbohydrate tolerance test (October 25 to November 6) the plasma bicarbonate barely rose above the minimum normal (53 volume per cent. CO_2), never approaching the average normal (65 volume per cent.), and on the maintenance diet (November 8 to 10) it again fell just below 53. (From October 15 to November 10, 1915.)

Group 4.—Case 2414, patient aged 17 years, diabetic one year, was severe in carbohydrate intolerance, and admitted with a dangerous degree of acidosis

(plasma CO_2 26.4 volume per cent.). The patient fasted for seven days before admission, following, he thought, the technic employed in this hospital, which he had experienced on two previous admissions.

The summary shows a case in severe acid intoxication (plasma CO_2 26.4 volume per cent.) on admission to the hospital, after a fast of seven days at home. The fast was continued for another day under hospital observation. This was followed by a further drop in the plasma CO_2 of 2.2 volume per cent., which, at this low level, with the onset of hyperpnea and his history of fasting at home, was taken as indication enough to interrupt the fast. The second day in the hospital the patient was put on a diet of lean meat and eggs, constituting 19 calories per kg. body weight. This was accompanied by sodium bicarbonate by mouth. The following morning the hyperpnea had vanished and the plasma CO_2 had risen to 34.3 per cent. The diet was gradually cut down during the next ten days and the bicarbonate therapy continued until the patient had a normal plasma CO_2. The subsequent fast of five days made the patient aglycosuric and cleared up the ketonuria (Gerhardt reaction).

A carbohydrate tolerance test was given after the fasting period. This was so low (only 30 gm. of carbohydrate when green vegetables alone were given) that a second carbohydrate tolerance test was attempted, as it was thought probable that the severe acidosis present on admission had in some way mitigated against a higher tolerance. The second test confirmed the first.

The maintenance diet was then gradually built up to 1,550 calories (39 calories per kg. body weight), which included 2 gm. of protein per kg. body weight. No carbohydrate was tolerated on mixed diet.

The patient died a year later (March, 1917) in coma. (From February 19 to March 21, 1916.)

Individual diabetics vary greatly, not only in their ability to metabolize food without glycosuria, but also in their tendency to develop acidosis. Fasting decreases this tendency in most patients, but greatly exacerbates it in a minority. It has been found that it is possible to divide diabetic patients into four groups, according to their tendencies to develop acidosis, and that more or less typical treatment is required for the members of each group.

In this paper are described the methods employed at the hospital of The Rockefeller Institute to detect as early as possible the development of acidosis and to prevent its progress and typical examples are given of the procedure and results with patients of each group.

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THE OCCURRENCE OF BACILLUS INFLUENZÆ IN THE NORMAL THROAT.

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The present study is a continuation of the work of Pritchett and Stillman on the occurrence of *Bacillus influenzæ* in throats and saliva, with additional observations upon the media used and a study of the distribution of *Bacillus influenzæ* among the personnel of two institutions.

Pritchett and Stillman[1] found that of 177 persons who gave no history of having had influenza, 74, or 42 per cent, harbored *B. influenzæ* in their throats during November and December of 1918. The total incidence of influenza bacillus carriers among 231 normal and late convalescent individuals was 99, or 43 per cent. From 49 cases of uncomplicated influenza, *B. influenzæ* was recovered in 41 instances, or 83 per cent, while from 43 cases complicated by bronchopneumonia this organism was cultivated in 40, or 93 per cent. Of 6 cases of bronchopneumonia, which were probably late cases of influenza, all showed influenza bacilli. The incidence of *B. influenzæ* was much lower in 20 cases of lobar pneumonia, as only 11 cases, or 55 per cent, were positive. Pneumococci of Types III and IV, which are the groups usually encountered in normal mouths, were the types of pneumococci which were recovered from the cases of influenza complicated with bronchopneumonia.

Methods.

As in the previous study, the medium used in this investigation for the isolation of *Bacillus influenzæ* was Avery's[2] oleate hemoglobin agar. 5 cc. of a 2 per cent solution of neutral sodium oleate were added to 95 cc. of meat infusion agar which made a final concentration of oleate of 1:1,000. 1 cc. of a suspension of rabbit red blood cells was then added while the agar was still hot. Plates were poured

[1] Pritchett, I. W., and Stillman, E. G., *J. Exp. Med.*, 1919, xxix, 259.

[2] Avery, O. T., *J. Am. Med. Assn.*, 1918, lxxi, 2050.

containing about 15 cc. each. The medium was made fresh each day as the surface soon became dry.

The optimum hydrogen ion concentration for the isolation of influenza bacillus from the throat seems to be between pH 7.2 and 7.5. In every test of medium which was more alkaline than that having a pH of 7.5, unsatisfactory results were obtained. In one instance cultures were made on agar at pH 7.9 from 12 individuals who previously had been persistent carriers. Though the cultures were repeated, only 2 showed small colonies of *Bacillus influenzæ*. But, when repeated a third time on medium with a pH of 7.5, 8 were found

TABLE I.

Comparison of Cultures upon Media of Different Hydrogen Ion Concentrations.

Culture No.	pH 8.0		pH 7.4	
	Series 1.	Series 2.	Series 1.	Series 2.
1	−	−	++	++
2	−	−	++	++
3	−	−	−	−
4	−	−	−	−
5	−	+	++	+
6	−	−	+	++
7	−	−	++	+
8	−	+	++	+
9	−	−	++	++
10	−	−	−	++
Total positive.	0	2	7	8

+ indicates colonies of *B. influenzæ* present; ++ many colonies of *B. influenzæ* present.

to be carrying influenza bacilli. Of 36 other individuals chosen at random, 14, or 38 per cent, were positive when agar at pH 7.4 was used, but only 4, or 11 per cent, with agar at pH 7.9. In Table I are given the results obtained in an examination of 10 individuals from whom both positive and negative plates had previously been obtained. Cultures were taken twice during the same day upon medium at pH 8.0 and at pH 7.4. No positive plates were found in Series 1 on medium with a pH of 8.0, but in Series 2, 2 plates contained several small colonies. On the other hand, Series 1 with 7.4 pH agar shows 7 positive plates, and Series 2, 8 positive plates.

Negative results are so consistently obtained with medium having a reaction above pH 7.5 that one is led to question the value of all negative results obtained upon medium with an unknown hydrogen ion concentration.

All cultures here reported, unless otherwise stated, were taken from the posterior wall of the pharynx. As shown by Table II cultures from the tonsils proved to be unsatisfactory. In a series of 77 individuals, 3 cultures were made from the throat—1 culture from each tonsil, and 1 from the posterior wall of the pharynx. In 4 individuals positive cultures were obtained on plates from all three sources. In 4 other individuals the cultures from the pharynx and from one or the other tonsil were positive. In 11 individuals only the culture from

TABLE II.

Comparison of Cultures from the Pharynx and the Tonsils.

Source of cultures.	No. positive.
Pharynx and both tonsils	4
" " one tonsil	4
Only tonsil	0
" pharynx	11
Total No. of cases positive	19
" " " " negative	58
" " " " examined	77

the pharynx showed a growth of influenza bacilli. In no case were the organisms isolated from the tonsil and not from the pharynx. The influenza bacillus can unquestionably be isolated from the tonsils, but as there are so many other colonies on the plates made from the tonsils, the influenza bacilli are either overgrown or the colonies are so small that they are hard to detect. West tubes used in a few instances did not give any more satisfactory results than did the simpler method of pharyngeal cultures. Hence, it seemed unnecessary to subject the large group of individuals studied to the discomfort of nasopharyngeal cultures.

All organisms designated as *Bacillus influenzæ* were demonstrated to possess the typical morphological and cultural characteristics of this organism; all cultures were proved to be definitely hemoglo-

binophilic. With the exception of staphylococci and diphtheroids, only Gram-negative organisms were encountered upon the oleate hemoglobin agar plates. In addition to the influenza bacillus, there appeared on these plates various types of Gram-negative cocci of the *Micrococcus catarrhalis* group and Friedländer's bacillus group, and also the unidentified bacillus, described by Pritchett and Stillman, which is differentiated from *Bacillus influenzæ* by its hemolytic properties.

RESULTS.

Cultures were taken once a month for a period of 6 months from the throats of the personnel of the Laboratories and the Hospital of

TABLE III.

Incidence of B. influenzæ in Convalescent and Normal Individuals.

	Nov. and Dec., 1918.			Jan., 1919.			Feb.			Mar.			Apr.			May.		
	No.	Positive cases.		No.	Positive cases.		No.	Positive cases.		No.	Positive cases.		No.	Positive cases.		No.	Positive cases.	
		No.	Per cent.		No.	Per cent.		No.	Per cent.		No.	Per cent.		No.	Per cent.		No.	Per cent.
Normal individuals	177	74	42	127	17	13	122	42	34	109	56	51	96	33	34	86	34	39
Convalescents	54	25	46	42	1	2	40	16	40	42	19	45	38	15	39	28	13	46
Total	231	99	43	169	18	11	162	58	36	151	75	50	134	48	36	114	47	41

The Rockefeller Institute in accordance with the method outlined above. It has been possible to follow but 84 of the original 231 individuals used in the earlier study. Of these, 15 have never yielded a positive result. 9 of 35 individuals positive upon the original examination have never since shown the presence of *Bacillus influenzæ* in the throat. Of the total 84, 6 have been positive for 5 months and 13 for 4 months.

During the months from December, 1918, to June, 1919, as shown in Table III, the percentage of carriers in a group of approximately 150 individuals has been 43, 11, 36, 50, 36, and 41, respectively. In the previous study the individuals who gave a history of having had

influenza during the time of the epidemic were classed as convalescents. These same individuals have been followed during the complete period. 46 per cent were positive in November and December, 1918, 2 per cent in January, 1919, 40 per cent in February, 45 per cent in March, 39 per cent in April, and 46 per cent in May.

As previously mentioned, any culture medium which varies from the optimum hydrogen ion concentration is unsuitable for use in the isolation of *Bacillus influenzæ* from the throat. The reaction of the medium used in November and December had a pH of 7.2, while that for March, April, and May had a pH of 7.4 and 7.5. The reaction of the medium employed during January and February was not tested, and it is apparent, therefore, that no importance can be attached to the low incidence during January.

TABLE IV.

Incidence of B. influenzæ in Cases of Acute Respiratory Infections.

Date.	No.	Positive.	Per cent positive.
1919			
Feb.	9	5	55
Mar.	16	13	81
Apr.	12	8	67
Total.	37	26	70

As is seen from Table IV, *Bacillus influenzæ* was recovered from 70 per cent of the persons suffering from acute respiratory diseases who were admitted to the Hospital of The Rockefeller Institute during the months of February to April. Thus it is seen that during the same months a larger percentage of positive cultures was obtained from patients suffering from acute respiratory diseases than from normal individuals.

During the latter part of January, an epidemic of influenza occurred in a girls' school. While the pandemic of influenza raged in the country surrounding this institution during the fall of 1918, a strict quarantine was maintained and only a few questionable cases developed. While the institution was still under strict quarantine, a teacher who had spent Sunday at home developed influenza. The

first cases developed in her classes, and within 10 days over half the students were ill. The epidemic then subsided as suddenly as it arose. On February 16 throat cultures were taken from 52 girls who were still in the infirmary. Of these, 20, or 38 per cent, showed *Bacillus influenzæ*. In one cottage which had been under quarantine on account of trachoma since October, 1918, no cases of influenza developed. Of the 20 girls in this cottage, 5, or 25 per cent, showed *Bacillus influenzæ*.

In a boys' orphan asylum where a lax quarantine had been maintained and which gave no history of an epidemic of influenza, 190 throat cultures were taken from boys whose ages varied from 10 to 12 years. *Bacillus influenzæ* was recovered from 74, or 39 per cent.

DISCUSSION.

Whatever may be the etiologic relationship of *Bacillus influenzæ* to epidemic influenza, there is little doubt of the significance of this organism as a secondary invader in this type of respiratory infection. Facts, therefore, concerning the distribution, occurrence, and persistence of *Bacillus influenzæ* in the secretions of the respiratory tract are of importance in epidemiological investigation and in prophylaxis. In a study of the occurrence of *Bacillus influenzæ* in throats and saliva during the epidemic of influenza last fall, Pritchett and Stillman found this organism present in 42 per cent of 177 healthy persons from whom no history of respiratory infection was obtainable. These observers found the same organism in the throats of convalescents from influenza in 46 per cent of individuals studied. In the same epidemic period Lord, Scott, and Nye,[3] by cultural methods, demonstrated influenza bacilli to be present in the pharyngeal secretions of 76 per cent of 34 healthy men of the Harvard Student Army Training Corps. Opie and his collaborators,[4] by cultural and mouse inoculation methods, found *Bacillus influenzæ* in the mouths of 35.1 per cent of all healthy men examined at Camp Funston.

[3] Lord, F. T., Scott, A. C., Jr., and Nye, R. N., *J. Am. Med. Assn.*, 1919, lxxii, 188.

[4] Opie, E. L., Freeman, A. W., Blake, F. G., Small, J. C., and Rivers, T. M., *J. Am. Med. Assn.*, 1919, lxxii, 108.

These figures serve to indicate the wide distribution and prevalence of the organism during the severe epidemic of this acute respiratory disease.

Since the group of individuals studied by Pritchett and Stillman comprised for the most part the personnel of The Rockefeller Institute, it has been possible to make repeated cultural examinations of the throats of 84 of the same persons during the 6 months subsequent to the original observation. From this study certain facts of interest have been acquired concerning the duration of the carrier state, and the relative frequency of this organism in normal throats after the subsidence of the epidemic. The present study indicates that the percentage incidence of those harboring *Bacillus influenzæ* in the upper respiratory tract is as great during the postepidemic period as it was during the influenzal epidemic. During December, 1918, to June, 1919, the percentage of carriers in a group of 150 individuals has averaged 41 per cent per month. This percentage incidence of healthy persons found to harbor influenza bacilli in their throats and saliva is approximately the same as that recorded by Pritchett and Stillman during the height of the epidemic. In addition, it is of interest that in a boys' orphan asylum in which no case of influenza had occurred during the epidemic, 39 per cent of throat cultures taken from 190 boys showed the presence of *Bacillus influenzæ*. This percentage incidence of positive cultures is the same as that found in the examination of 52 convalescents from influenza in an institution for girls in which over half the personnel had suffered from the disease.

Furthermore, it is evident that *Bacillus influenzæ* may persist in the throats of healthy carriers for a considerable period of time. In 6 instances individuals have had positive cultures on repeated examinations during 5 months, and in 13 other instances during a period of 4 months. In chronic non-tuberculous disease of the lungs *Bacillus influenzæ* is known to persist for long periods of time in the secretions of the respiratory tract. The present study, however, demonstrates that in the absence of a focus of infection this bacillus may dwell on apparently normal mucous membranes for long intervals. Whether the organisms thus encountered represent a variety less parasitic than strains actually associated with pathologic lesions

is a problem requiring greater knowledge concerning the biologic differentiation of bacilli of the hemophilic group than is now available.

The technical difficulties incident to the isolation of *Bacillus influenzæ*, particularly from the mixed flora of the upper respiratory tract, have been materially lessened by the use of oleate hemoglobin agar. The optimum hydrogen ion concentration for this medium is shown to lie between pH 7.2 and 7.5. Any variation beyond this range, particularly on the alkaline side, reduces markedly the suitability of this medium for growth of *Bacillus influenzæ*. Oleate hemoglobin agar not only enhances the growth of the influenza bacillus, but by inhibiting the growth of other organisms, such as streptococci and pneumococci, greatly facilitates the isolation of *Bacillus influenzæ* from a focus which harbors a wide variety of other bacteria.

CONCLUSIONS.

1. The frequency of occurrence of *Bacillus influenzæ* in the throats of normal individuals has been as great in the period subsequent to the epidemic of influenza as it was during the period of the epidemic.

2. *Bacillus influenzæ* may persist in the throats of healthy carriers for a considerable period of time.

3. A considerable proportion of individuals not exposed during the epidemic has been found to carry *Bacillus influenzæ* in their throats.

4. Oleate hemoglobin agar has been found to be a very satisfactory selective medium for cultivation of *Bacillus influenzæ* from the throat. To give optimum results it should have a reaction of from pH 7.2 to 7.5.

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THE DETERMINATION OF CARBON MONOXIDE IN BLOOD.

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The determination of carbon monoxide in blood may be performed by gasometric, colorimetric, or spectrophotometric methods.

Previous gasometric methods have been laborious and most of them required special apparatus (as those of Gréhant, de Saint Martin, Nicloux, and others). The method employed by Zuntz and Plesch (1) seems to be the easiest. They determine the carbon monoxide in 1 cc. of blood, using ferricyanide to set the gases free (CO and O_2) and burn the CO to CO_2, which is absorbed by KOH. They calculate the amount of CO by the difference in pressure as in Haldane and Barcroft's method for blood gas analysis. According to the authors, the expulsion of the blood gases is finished after 1 hour.

The colorimetric method of Haldane (2) is based upon the fact that dilute carmine solutions have nearly the same color as dilute solutions of carbon monoxide hemoglobin. If it is known how much carmine has to be added to normal blood in order to give it the same color as blood completely saturated with carbon monoxide, and it is determined how much of the carmine must be added to normal blood to give it the color of the blood with the unknown content of CO, a simple calculation by proportion will give the degree of saturation with carbon monoxide.

This method seems to be very accurate in Haldane's hands, while others, like Krogh (3), and Zuntz and Plesch (1), have been unable to obtain good results with it. Plesch (4) has modified it, using a hemoglobin solution saturated with carbon monoxide instead of carmine for the titration.

Spectroscopic methods have been used by Hüfner and later by Hartridge (5). Krogh (6) has recently modified the method of the latter and used it for the determination of the oxygen capacity of

very small amounts of blood; his method is still rather rough, but he hopes to make it more accurate with finer instruments.

An easy gasometric method seems still to be needed and we have worked out one which permits the determination of the carbon monoxide gasometrically in 2 cc. of blood in the course of 10 to 15 minutes. We have employed this method for the determination of the blood volume and for the study of the action of carbon monoxide on blood.

The Method.

The principle of our method is to set free the oxygen and carbon monoxide from their combination with hemoglobin in the blood by addition of ferricyanide and then to remove both gases with the help of a Torricellian vacuum in the Van Slyke apparatus for blood gas analysis. The oxygen is absorbed in the apparatus by alkaline pyrogallate and the volume of residual carbon monoxide is measured directly at atmospheric pressure, a correction being made for the small and constant amount of nitrogen gas physically dissolved by blood.

The procedure is, up to the time when the expelled gas is measured, exactly the same as that for the oxygen method described by Van Slyke (7), and it is therefore unnecessary to repeat it here; the same amount of blood and the same solutions are used, and only the shaking has to be continued a little longer before a constant reading is obtained. This takes about 2 to 3 minutes and is a little different for different species of blood; it probably depends upon the facility with which the blood is laked.

When the reading of the volume of the gas mixture, consisting of oxygen, carbon monoxide, and a little nitrogen, is constant, a solution of alkaline pyrogallate[1] is introduced into the cup of the apparatus, is covered by a thin layer of paraffin oil, and is allowed to flow slowly down the inner wall of the graduated part of the apparatus. A little suction is produced during this part of the procedure by lowering the leveling bulb slightly.

The absorption of the oxygen is very rapid and is completed in less than 1 minute; the reading is taken and the pyrogallate solution intro-

[1] Prepared by dissolving 10 gm. of pyrogallic acid in 200 cc. of strong potassium hydroxide (160 gm. of KOH dissolved in 130 cc. of water).

duced once more until a constant reading is obtained. The gas is then measured under barometric pressure in the same way as described by Van Slyke for carbon dioxide (8) and oxygen.

As the solution is very dark and it is a little difficult to get good readings of the meniscus, we have produced a new meniscus by letting a little water flow down after the pyrogallate solution; the water floats on the top of the fluid and one can get readings to about 0.002 cc. Instead of water a few drops of octyl alcohol may be used.

The apparatus is washed out twice with dilute ammonia solution after each determination.

Calculation.—The gas measured is reduced to standard conditions by multiplying by the factor $(0.999–0.0046\ t) \times \frac{\text{Barometer}}{760}$, t being the temperature in °C. If 2 cc. of blood have been used, the values of this factor in Column 3 of Table I of Van Slyke's paper on oxygen[2] may be used, the result then being expressed in cc. of CO per 100 cc. of blood, when the nitrogen correction, 1.2 cc., is subtracted.[3]

EXPERIMENTAL.

Air was analyzed in the Van Slyke apparatus in order to find the best way of absorbing the oxygen. When the pyrogallate solution was introduced in the manner described above, the oxygen was absorbed in 30 seconds.

The method was tried in the following way. As the oxygen and the carbon monoxide replace each other in the combination with hemoglobin and the oxygen capacity equals the carbon monoxide capacity, blood with known percentages of carbon monoxide may be obtained by mixing different amounts of blood saturated with carbon monoxide with blood saturated with air. By taking, for instance, one part of CO blood and four parts of O_2 blood, the analysis should give 20 per cent CO and 80 per cent O_2.

Table I shows the amounts of oxygen and carbon monoxide found by analysis and the amount calculated.

[2] Van Slyke, D. D., *J. Biol. Chem.*, 1918, xxxiii, 130.

[3] The nitrogen correction is 1.2 per cent, instead of the calculated value 0.9 per cent, when actually determined by Bohr and by ourselves.

It was seen from Table I how closely the found values agree with those calculated, except in No. 8, where the discrepancy is 2.7 vol-

TABLE I.

No.	Blood used.	Found.			Calculated.			Kind of blood.
		Oxygen.	CO	Proportion of hemoglobin saturated with CO.	Oxygen.	CO	Proportion of hemoglobin saturated with CO.	
	cc.	*cc.*	*cc.*	*per cent*	*cc.*	*cc.*	*per cent*	
1	2	0.212	0.206	50.7	0.209	0.209	50.0	Guinea pig.
2	2	0.208	0.210	49.8	0.209	0.209	50.0	Same blood.
3	2	0.118	0.240	32.9	0.117	0.238	33.3	Rabbit.
4	2	0.170	0.328	34.1	0.116	0.332	33.3	Ox.
5	2	0.088	0.250	26.0	0.084	0.252	25.0	Rabbit.
6	2	0.128	0.390	24.5	0.129	0.388	25.0	Ox.
7	2	0.068	0.240	22.1	0.062	0.246	20.0	Rabbit.
8	2	0.054	0.224	19.43	0.046	0.231	16.7	"
9	4	0.091	0.434	17.3	0.088	0.437	16.7	Same blood.

umes per cent. By taking 4 cc. of the same blood instead of 2 cc., this error is brought down to 0.6 volume per cent. We therefore recommend the use of a little more blood, 3 or 4 cc. (and the correspondingly increased amount of ammonia), for analysis, if the percentage saturation of CO is very small and the actual amount of CO found in 2 cc. of blood is 0.05 cc. or less.

In two rather rough experiments, two guinea pigs were given illuminating gas under a bell jar until they fell unconscious. They were then taken out and blood was drawn by heart puncture for analysis. One of them died while the blood was being taken; the other recovered and behaved normally an hour afterwards.

TABLE II.

	O_2	CO	Saturation with CO.	Remarks.
	cc.	*cc.*	*per cent*	
Guinea Pig 1.....	0.115	0.353	75.4	Died during bleeding.
" " 2.....	0.080	0.258	76.3	Recovered.

Table II shows that a guinea pig can recover after carbon monoxide poisoning, even when the blood is 76.3 per cent saturated with carbon monoxide.

SUMMARY.

A method is described for the determination of carbon monoxide in blood, the technique of which is exactly the same as that previously described by Van Slyke for the determination of oxygen, except that after the gases are extracted the oxygen is absorbed in the apparatus by introducing alkaline pyrogallate solution. The carbon monoxide remains and is measured directly at atmospheric pressure.

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THE DETERMINATION OF BLOOD VOLUME BY THE CARBON MONOXIDE METHOD.

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There is still much confusion regarding the exact value of the blood volume in human beings, though much work has been done in this field of investigation. Values for the blood volume for men are given, ranging from $\frac{1}{21}$ to $\frac{1}{8}$ of the body weight. These widely differing results have been obtained by the use of different methods.

There are one direct and several indirect methods for the determination of the blood volume.

Direct Method.—Welcker (1) in 1854 was the first one to determine the blood volume by a method which still is regarded as the standard; he bled animals to death, washed out the vessels with water, and extracted the hemoglobin still remaining in the tissues by mincing the organ minus the bile and the content of the bowels, and placing them in water for several days. By comparing the hemoglobin content of the first blood and the blood washings and extracts brought together, he found the blood volumes of mammals to constitute $\frac{1}{13}$ of the body weight. The same value was obtained for human beings by Bischoff (2), who used this method on two criminals.

Welcker's method has been modified and improved by several investigators, but the general principle is the same as in 1854. The results obtained in animals with this method have differed because of incomplete washing and extraction, and the use of inexact methods for the hemoglobin determinations.

Indirect Methods.—These methods must be used in experiments on living animals. There are various principles for the indirect determination of the blood volume. The best of them may be divided into two groups: (1) A known amount of an easily determinable substance, which is kept within the circulatory system for a sufficiently long time for thorough mixing, is introduced into the blood and the concentration of it determined. (2) The blood is either (*a*) diluted, or (*b*) concentrated in various ways, and the blood volume calculated from the variation in the content of hemoglobin or corpuscles.

Group 1. (*a*) *Carbon Monoxide Method.*—The principle of this method is to administer a certain amount of carbon monoxide gas to the individual, and then

to determine the degree of saturation of the blood with CO or the actual amount of CO per unit of blood. It was first used by Gréhant and Quinquaud (3), and later by Haldane and Smith (4), Oerum (5), Douglas (6), Boycott and Douglas (7), and Plesch (8). (*b*) The dye method of Keith, Rowntree, and Geraghty (9) is the method most widely used for the present in clinical work. A dyestuff, vital red, is injected intravenously and determined colorimetrically in the blood. The relative amounts of plasma and corpuscles are determined by the hematocrit method. (*c*) The antitoxin method of von Behring (10) is based upon the observation that tetanus antitoxin remains in the circulation for a long period; a known amount is injected, and the concentration then determined in the blood. (*d*) The acacia method of Meek and Gasser (11) has been tried only in animals so far, and the experience is not large enough to judge of its utility. Acacia is injected and determined in the blood as furfurolphloroglucine.

Group 2. (*a*) *Dilution Methods.*—The only one of these methods which has withstood criticism is based upon the observation of Cohnstein and Zuntz (12) that isotonic sodium chloride solutions are kept in the circulation for a relatively long time, and diffuse very slowly out into the tissues; this is utilized for the determination of the blood volume, the red cells being counted before and after infusion. Plesch (8) seems to have developed this method to further exactness by determining the hemoglobin instead of the cells with the help of his chromophotometer. As the variations in the cell or hemoglobin content obtained by dilution hardly exceed 10 per cent, the exactness of the method evidently depends on how accurately these constituents of the blood can be determined.

(*b*) *Concentration Methods.*—The method of Tarchanoff (13), who determined the hemoglobin before and after a steam bath and the decrease in weight through loss of water, and calculated the blood volume from these two factors, has been justly criticized and cannot be relied upon, as the water may be derived from other sources in the body than the blood.

Quincke (14) transfused blood with a certain amount of red corpuscles to two anemic patients and calculated the blood volume from the increase in the red count. Lindeman (15) uses the same principle. These methods can only be used in anemia.

Of these methods only three are of practical value for physiological and clinical purposes; they are the carbon monoxide method, the infusion method of Cohnstein and Zuntz, and the vital red method.

The results obtained by these methods vary. In animals, both the carbon monoxide method and the infusion method have given nearly the same values as the Welcker method; Gréhant and Quinquaud (3) found in nine dogs values from $\frac{1}{11}$ to $\frac{1}{13.8}$ of the body weight with the CO method, which corresponded to Welcker's own results in dogs. Douglas (6) found a close agreement between the CO method and the bleeding method in five rabbits; Boycott and Douglas (7), repeating the experiments later, found a little higher value with the CO method (2 per cent). Plesch (8), in dogs, tried subsequently the CO, the infusion, and

the bleeding method, and the results were uniform. The vital red method has never been checked up by the Welcker method as far as can be seen from the literature.

In human beings the results are widely different. Bischoff's values, $\frac{1}{13}$ of the body weight, were regarded as the standard until Haldane and Smith (4), with the CO method and carmine titration, in fourteen normal men found $\frac{1}{21}$, the highest value being $\frac{1}{16}$ and the lowest $\frac{1}{30}$ (in a very fat man). Oerum (5), using the same technique, found in men $\frac{1}{19.2}$, and in women $\frac{1}{21.8}$. Plesch (8), using a gasometric method for the CO determination in four men, found the average ratio $\frac{1}{17.9}$; also in five persons, some of whom were reported to be fat, he determined the blood volume with the infusion method and found $\frac{1}{19.1}$. Bischoff's results, therefore, seem to be too high, inasmuch as the two criminals were hardly normal individuals, one at least suffering from scurvy. The method used is also open to criticism.

Douglas (16), in 1910, made a series of determinations with the CO method on himself and another subject with Haldane's technique, but waited a longer time before he took the blood sample for analysis. These results show a mean value of $\frac{1}{13.9}$ for his own and $\frac{1}{12.5}$ for the other subject, values more in accordance with those of Bischoff. He found errors in Haldane and Smith's determinations due to incomplete mixing of the blood, as the blood sample was taken too early after the breathing of the carbon monoxide.

In 1915 Keith, Rowntree, and Geraghty (9), with their vital red method, found still higher values, the mean in normal men being $\frac{1}{11.8}$ of the weight. This method has never been controlled by the Welcker method as far as can be seen, but the authors show its reliability toward relative changes by drawing a certain amount of blood and finding a corresponding drop in the blood volume.

It may be seen from this review of the literature how uncertain is our knowledge of the blood volume in human beings. Since the carbon monoxide method in animals has given satisfactory results as compared with the standard method of Welcker, and in human beings the results obtained by the various investigators have differed widely, it seems worth while to make further investigations in this field, especially since the technique used before has been rather difficult.

It seems certain, according to Douglas, that Haldane and Smith's

figures are too small, and so must be the figures of Oerum, as he used the same technique. There remains, therefore, only the determinations of Plesch in four persons, giving the average of $\frac{1}{17.9}$ and those of Douglas, on himself and another man, giving the values of $\frac{1}{13.9}$ and $\frac{1}{12.5}$ of the body weight.

The carbon monoxide method has been criticized by Dreyer (17) and his coworkers, who, in rabbits, used Haldane and Smith's technique, and got so much divergence in the figures that they concluded it could not be used in its present form. They therefore determined the blood volume by injecting in rabbits' blood a known amount of agglutinin, determined the percentage in the serum, then washed out the circulation, and determined the percentage of agglutinin in the washing. They claim the blood volume to be a function of the surface area, so that, for instance, smaller rabbits have a relatively higher blood volume than the larger ones.

The adverse criticism of the carbon monoxide method may be due to the difficulty of the technique, as the carmine titration of Haldane requires long training and a highly developed color sense. All the carbon monoxide determinations in the present paper are performed with the help of the gasometric method, described in the preceding paper, which makes the whole technique much simpler and fitted for general use.

Blood Volume Determinations in Animals.

The results of numerous determinations of Boycott and Douglas (7) show that rabbits' blood constitutes from $\frac{1}{22}$ to $\frac{1}{18.1}$ of the body weight, as determined both with the carbon monoxide and the washing out method. The average of 52 rabbits with the washing out method was $\frac{1}{20.9}$ or 4.77 cc. of blood per 100 gm. of body weight, while the carbon monoxide method (with the carmine titration) gave a little higher result, $\frac{1}{18.1}$ or 5.5 cc. per 100 gm.

Methods.

The arrangement used was that described by Douglas (6). It is therefore unnecessary to repeat it here.

The principle is to let the rabbit breathe into a closed system which is supplied with arrangement for removing the carbonic acid and renewing the oxygen. A measured amount of carbon monoxide is introduced into the apparatus, and 10 minutes after the entire amount is given a sample of blood is drawn from the ear vein and analyzed for carbon monoxide. At the same time a sample of the air in the chamber is taken for determination of the O_2, CO_2, and CO. The blood volume is calculated from the amount of carbon monoxide absorbed by the animal and the concentration of it in the blood. As in Douglas' experiments, a tube connected with a bell jar, partly immersed in a glass of water, was introduced into the respiratory chamber, and served as an indicator of the pressure in the apparatus. While the air sample was drawn, the oxygen current was cut off and the water allowed to rise in the bell jar by raising the glass in order to compensate for the negative pressure produced by the sucking out of the air.

The capacity of the apparatus (the chamber and the air in the rubber tubing, the pump, and the bell jar) was 750 cc., a little larger than that of the apparatus employed by Douglas (6), and Boycott and Douglas (7).

The carbon monoxide was prepared by heating oxalic acid and concentrated sulfuric acid, and the gas was collected over water made alkaline with sodium hydroxide, with which the gas was shaken in order to get rid of the CO_2. The gas was analyzed every 2nd day by shaking it in a Hempel pipette with cuprous chloride solution, which absorbs the carbon monoxide and O_2, and from the amount of nitrogen left the air content of the gas was calculated. The gas first evolved was discarded. The carbon monoxide content of the rest was 95 to 98 per cent.

The carbon monoxide in the blood was determined by the method described in the preceding paper, the blood, 4.5 cc. in all, being drawn from the ear vein without stasis and kept under paraffin oil; 2 cc. were used for each analysis. The hemoglobin was determined

by the Palmer method (18). The air in the chamber was analyzed for O_2 and CO_2 in the Haldane-Henderson apparatus.

The determination of the carbon monoxide left in the chamber could not be made by gasometric methods, and the method of Haldane (19) was employed, the principle of which is to shake the carbon monoxide-containing air with blood, to estimate colorimetrically the percentage of saturation of the blood with carbon monoxide, and deduce from this value, and the percentage of O_2 present, the percentage of carbon monoxide in the air. As bloods of different species and also of different individuals show different dissociation curves for the carbon monoxide hemoglobin, as shown by Krogh (20) and Haldane (21), blood from a single sheep was used, in which the dissociation curve was previously determined (preceding paper). The dissociation curve is a hyperbola of the formula

$$\frac{(O_2 \text{ percentage in air})}{(CO \text{ percentage in air})} \times \frac{\text{Hb CO}}{\text{Hb O}_2} = K$$

In the blood used $K = 179$ at 24°.

For the determination of the dissociation curve of the blood of our sheep, 5 cc. samples of the blood were rotated in bottles of known capacity (approximately 1 liter), filled with air plus known amounts of carbon monoxide. The blood was first placed in the bottle, which was then closed by a stopper containing a three-way capillary cock. A known volume (1 to 4 cc.) of analyzed CO gas was then forced in from a micro-gas-burette, in which the volume delivered could be read over mercury to within 0.002 cc. The tubes of the cock were filled with the CO before the measured amount was admitted into the bottle, so that errors due to dead space were avoided. The bottle with the blood and gas mixture was rotated for 2 hours at 24°C. Trial showed that equilibrium was obtained in this time. Samples of 2 cc. of blood were then withdrawn and used for the determination of O_2 and CO as described in the foregoing paper. The volume of CO taken up by the 5 cc. of blood was subtracted from the volume of CO originally added, in order to estimate the amount left in the gas phase. The oxygen concentration remained that of atmospheric air.

The results obtained in four determinations are shown in the curve of Fig. 1. The curve is the hyperbola plotted from the formula, with 179 taken as the value of K; the crosses represent the results experimentally obtained.

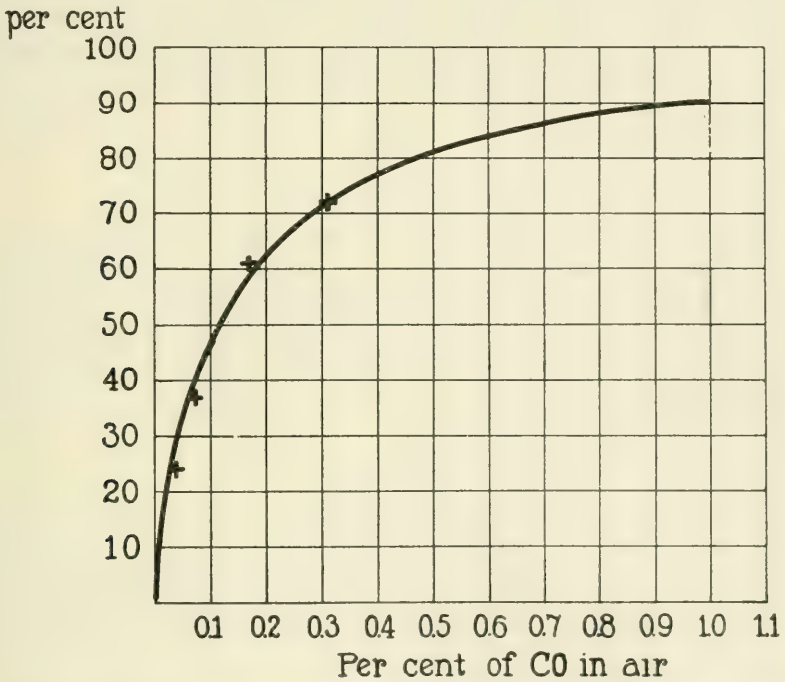

FIG. 1. The curve is the hyperbola plotted from the formula, with 179 taken as the value of K; the crosses represent the results experimentally obtained.

The value of K being known, small percentages of CO in air could be ascertained by shaking the latter with a known volume of the blood and determining the HbO_2 and HbCO, the calculation being $\textit{per cent CO in air} = \frac{21.9}{179} \times \frac{HbCO}{HbO_2}$. In calculating the actual percentage of CO in the gas-sampling tube allowance was made for the amount of CO taken up by the blood with which the air was shaken. The colorimetric determination was made by the method employed by Plesch (8), which is very simple and which gave fairly

good results when used on blood with known percentages of CO. The principle is: Three samples of the same blood are saturated; No. 1 with air, No. 2 with CO, and No. 3 with the air containing the unknown percentage of CO. 0.05 cc. of each sample is diluted with 10 cc. of a 1 per cent solution of Na_2CO_3 in each of three small test-tubes of equal bore. No. 2 is added to No. 1 until the color is the same as in No. 3. If, for instance, equal color is obtained by adding 2.5 cc. of No. 2 to 5 cc. of No. 1, then the degree of saturation is $\frac{2.5}{2.5+5} = 33.3$ per cent.

Results.

In fourteen rabbits taken from the stock the blood volume was determined. In two of the female rabbits the values obtained were much higher than the average, and they later proved to be pregnant. They were placed in a separate group and the blood volume determined again, post partum. In some of the other rabbits the blood volume also was determined twice. The results are given in Tables I to V.

TABLE I.

Normal Male Rabbits.

Rabbit No.	Date.	Weight.	Hemoglobin.	Blood volume.	Relation to body weight.	Cc. per 100 gm.
	1919	*gm.*	*per cent*	*cc.*		
77	July 3	1,500	(?)*	79.4	$\frac{1}{19.1}$	5.20
93	" 8	1,900	(?)*	95.7	$\frac{1}{19.9}$	5.02
89	" 10	1,600	93.4	80.9	$\frac{1}{19.8}$	5.05
80	" 10	2,250	94.3	107.1	$\frac{1}{21}$	4.76
99	" 14	3,010	86.2	146.9	$\frac{1}{20.5}$	4.88
82	" 14	2,200	87.7	115.4	$\frac{1}{19.06}$	5.25
98	" 15	2,200	93.4	111.1	$\frac{1}{19.8}$	5.05

* Determinations lost because of an incorrect hemoglobin standard.

TABLE II.

Normal Female Rabbits.

Rabbit No.	Date.	Weight.	Hemoglobin.	Blood volume.	Relation to body weight.	Cc. per 100 gm.
	1919	*gm.*	*per cent*	*cc.*		
66	July 2	2,300	(?)*	122.2	1/18.8	5.30
76	" 7	1,250	83.3	50.8	1/24.6	4.06
88	" 9	1,960	86.2	100.6	1/19.4	5.14
83	" 11	2,630	76.9	129.2	1/20.3	4.93
87	" 11	2,320	51.4	127.5	1/18.2	5.49

* Determination lost because of an incorrect standard.

TABLE III.

Repeated Determinations.
Male Rabbits.

Rabbit No.	Date.	Weight.	Hemoglobin.	Total oxygen capacity.	Blood volume.	Ratio.	Cc. per 100 gm.
	1919	*gm.*	*per cent*	*cc.*	*cc.*		
93	July 8	1,900	(?)*	(?)*	95.7	1/19.9	5.02
	" 21	1,940	59.3	10.14	92.4	1/21	4.76
	" 3	1,500	(?)*	(?)*	79.4	1/19.1	5.20
77	" 18	1,540	75.0	13.87	72.9	1/21.1	4.74
	" 10	2,250	94.3	18.68	107.1	1/21.1	4.76
80	" 17	2,270	90.9	17.54	102.1	1/22.2	4.50
	" 14	3,010	86.2	21.08	146.9	1/20.5	4.88
99	" 18	3,070	74.1	22.33	162.1	1/18.9	5.29

* Determination lost because of an incorrect standard.

TABLE IV.

Repeated Determinations.
Female Rabbits.

Rabbit No.	Date.	Weight.	Hemoglobin.	Total oxygen capacity.	Blood volume.	Ratio.	Cc. per 100 gm.
	1919	*gm.*	*per cent*	*cc.*	*cc.*		
76	July 7	1,250	83.3	7.83	50.8	$\frac{1}{24.6}$	4.06
	" 15	1,350	66.6	8.30	66.8	$\frac{1}{20.2}$	4.95
81	" 11	2,320	51.4	12.12	127.5	$\frac{1}{18.2}$	5.49
	" 17	2,300	47.4	11.44	129.0	$\frac{1}{17.8}$	5.62

TABLE V.

Blood Volume in Pregnant Rabbits before and after Term.

Rabbit No.	Determination made.	Date.	Weight.	Hemoglobin.	Total oxygen capacity.	Blood volume.	Ratio.	Cc. per 100 gm.	Remarks.
		1919	*gm.*	*per cent*	*cc.*	*cc.*			
65	Before.	July 8	2,650	69.4	22.86	177.3	$\frac{1}{14.9}$	6.70	Young ones, July 15.
	6 days post partum.	" 21	2,290	76.3	15.24	108.3	$\frac{1}{19.4}$	5.14	
87	Before.	" 9	2,560	94.0	24.29	141.1	$\frac{1}{18.1}$	5.52	
	"	" 16	2,570	74.4	22.2	161.3	$\frac{1}{15.3}$	6.54	Young ones, July 20.
	11 days after.	" 31	2,350	76.3	15.59	110.5	$\frac{1}{21.2}$	4.72	

In Tables III and IV is introduced a column, "total oxygen capacity," the figures in which indicate all the oxygen with which the blood is able to combine, calculated from the blood volume and the hemoglobin percentage. In our hemoglobin standard 100 per cent equals 18.5 volumes per cent of O_2.

DISCUSSION.

The results are expressed in parts of the crude body weight, though this may vary for various causes, such as food intake, content of bowels, etc. The average value for the blood volume in seven male rabbits (eleven determinations, Tables I and III) is $\frac{1}{20.21}$ of the body weight, or 4.95 cc. per 100 gm. The average in five non-pregnant female rabbits (seven determinations, Tables II and IV) is $\frac{1}{19.9}$ of the weight, or 5.02 cc. per 100 gm. If the two determinations from Table V in rabbits post partum are added, the mean for all seven females is the same, $\frac{1}{19.99}$, or 5.0 cc. per 100 gm. The results are in accordance with those obtained by Boycott (7) and coworkers with the washing out method.

The repeated determinations in Tables III and IV show a fairly close agreement in some of the rabbits, while in Rabbits 99 and 76 there is a considerable difference. But the total oxygen capacity is nearly constant in these two rabbits. Rabbit 76 had increased in weight from 1,250 to 1,350 gm., and to get comparable values the oxygen capacity in the first determination has to be multiplied by $\frac{1,350}{1,250} = 1.08$, and this gives the value of 8.46 cc. which is very close to the value found the second time, 8.30 cc. The same phenomenon is seen in the pregnant rabbit, No. 87, of Table V. The two determinations before partus gave $\frac{1}{18.1}$ and $\frac{1}{15.3}$ of the weight, which is a large increase in the blood volume. The hemoglobin concentration dropped, however, so that the oxygen capacity of the total blood supply of the animal remained constant, the blood merely having become diluted in the interval between determinations. About a week after partus the blood volume was again normal.

The blood volume of the rabbit, therefore, seems to be able to change normally, probably in the way that fluid passes in and out through the capillaries, a phenomenon in analogy with what has been observed in human beings in shock. Rabbits 99 (Table III), 76 (Table IV),

and 87 (Table V), in which these changes in the blood volume and constancy of the total oxygen capacity were so marked that they cannot be due to experimental error, show the smallest volume the first time, when they were unexperienced and scared when put into the apparatus, and a larger volume the second time, when they were used to the procedure. The material is too small, however, to draw any conclusion on this point, but Douglas has observed the same phenomenon of changes in the volume and constancy of oxygen capacity.

Table V shows the well known fact that pregnant animals have a larger blood volume absolutely and relatively than non-pregnant. A week post partum the blood volume is restored to the normal value.

Blood Volume Determinations in Human Beings.

The arrangement of apparatus was nearly the same as that described by Haldane and Smith (4), except that an ordinary Wolff bottle, filled with sticks of potassium hydroxide,[1] was used for the absorption of the carbonic acid, as seen in Fig. 2, and the experiments lasted longer, the subjects breathing for 10 to 15 minutes after the entire amount of carbon monoxide was given. The oxygen was supplied from a cylinder as rapidly as was necessary in order to keep the amount of gas in the bag approximately constant. The estimation of the relative volume of air in the bag was facilitated by placing the bag horizontally with a scale behind it as an indicator of the degree of filling.

The apparatus was filled with carbon monoxide to the three-way stop-cock before the experiment was started. The volume of the connecting parts and the bag, filled with air to the mark, was 3,700 cc. For the calculation of the amount of carbon monoxide left the volume of the lungs must be added; this is about 3,000 cc., and the total volume of the air in which the carbon monoxide was distributed thus was 6,700 cc.

The blood, being assumed to constitute not less than $\frac{1}{19}$ of the body

[1] Potassium hydroxide was used because of the high solubility of the potassium carbonate formed during the experiment; this carbonate was washed out by rinsing the sticks with water now and then.

weight, the amount of carbon monoxide given to the subject was so calculated that the saturation of the blood with carbon monoxide would not exceed 20 to 25 per cent. For instance, with the body weight 70 kilos, hemoglobin 120 per cent, blood volume at least $\frac{70}{19}$

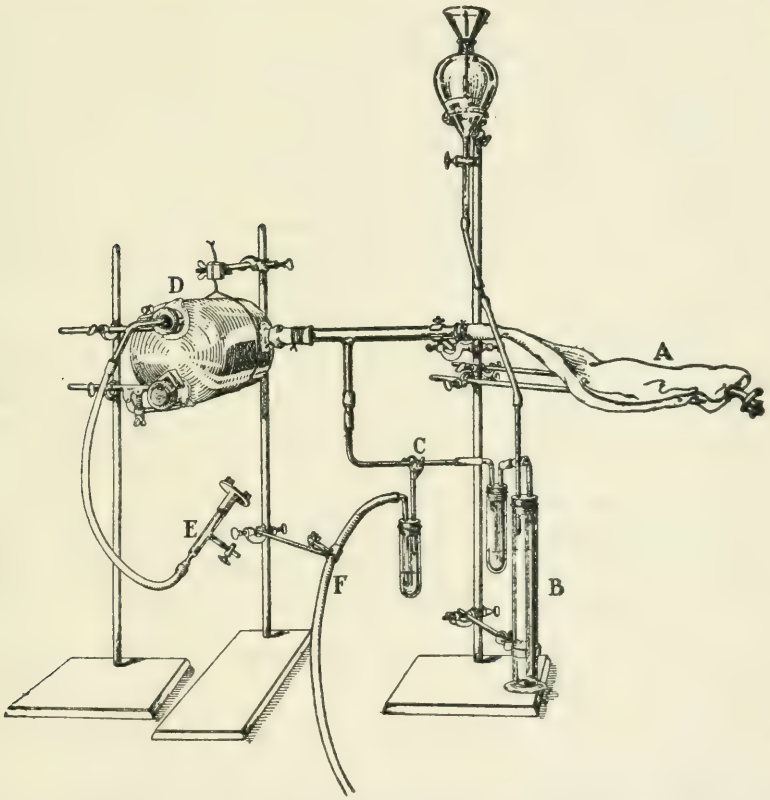

FIG. 2. A, rubber bag, B, volumetric measuring cylinder for carbon monoxide, C, three-way stop-cock, D, Wolff bottle for removing the carbon dioxide, E, mouthpiece, F, tubing connected with the oxygen cylinder.

= 3.7 liters, and as 100 per cent hemoglobin = 18.5 volumes per cent of O_2 capacity, the capacity in this case would be 22.2 cc. per 100 cc. of blood; per 3,700 cc. it accordingly would be 821.4 cc. One, therefore, could safely give 164 cc. of carbon monoxide reduced to standard conditions (760 mm. and 0°).

The blood sample was drawn without stasis from the arm vein and kept under paraffin oil. The carbon monoxide was determined by the method of Van Slyke and Salvesen,[2] 3 cc. being used for each determination, as the carbon monoxide content of the blood is rather small in these experiments.

The air in the bag was analyzed for oxygen, carbon dioxide, and carbon monoxide after each experiment, as in the animal experiments. In all except the last experiment the amount of carbon monoxide left was about 2 cc.: 1.47, 1.902, 2.53, 1.832, 1.96; average 1.938 cc. In the last experiment it was 4.42 cc., but in this case more oxygen was given than necessary, and the oxygen content of the bag was 33 per cent. This probably accounts for the slower absorption of the carbon monoxide, the dissociation curve of the carbon monoxide-hemoglobin being depressed when the oxygen percentage increases. If the experiments, therefore, are always performed in the same way, the amount of carbon monoxide left is constant, and if the apparatus has the same capacity as used in the present experiments, the correction which must be subtracted is 2 cc. The experiments done in this way are much simpler, the only determination which must be done being that of the carbon monoxide in the blood.

Six healthy individuals were examined, ranging in age from 23 to 37 years. The material was rather uniform as all were young people without any adipositas. The results are given in Table VI.

The average blood volume found was 3,888 cc., constituting $\frac{1}{16.8}$ of the weight, or 5.95 cc. per 100 gm. The extremes are $\frac{1}{14.3}$ and $\frac{1}{19.08}$. The largest volumes were found in Nos. 1 and 6, both of whom are tall and slim, especially No. 6 who is abnormally thin, and weighs much less than would correspond with his height. In No. 1 two determinations were made and the difference found is only 7 cc. of blood.

The mean value, then, is a little larger than that of Plesch, who found $\frac{1}{17.9}$ and smaller than that of Douglas, who found in two persons $\frac{1}{13.9}$ and $\frac{1}{12.5}$.

[2] Van Slyke, D. D., and Salvesen, H. A., *J. Biol. Chem.*, 1919, xl, 103.

All the persons experimented on felt comfortable, and did not have any disagreeable sensations. The breathing was easy, and even a certain degree of dyspnea is not likely to interfere with the use of this method in pathological cases. Former investigators have used the carbon monoxide method in heart, kidney, and anemic cases without any difficulties.

TABLE VI.

Blood Determinations in Human Beings.

No.	Date.	Name.	Age.	Weight.	Duration	Hemoglobin.	Blood volume.		
								Ratio.	Cc. per 100 gm.
	1919		*yrs.*	*kg.*	*min.*	*per cent*	*cc.*		
1	July 28	Dr. H. S.	30	68.8	32	114	4,594	$\frac{1}{14.97}$	6.67
	Aug. 1	"		68.8	35	114	4,601	$\frac{1}{14.82}$	6.74
2	July 29	Mr. A. S.	26	60.9	28	124.9	3,464	$\frac{1}{17.6}$	5.68
3	" 29	Dr. H. A.	36	66.4	23	118	3,479	$\frac{1}{19.08}$	5.23
4	" 30	Dr. V. S.	37	72.7	27	113	3,877	$\frac{1}{18.7}$	5.35
5	" 31	Dr. J. T.	28	51	23	114	3,429	$\frac{1}{17.7}$	5.65
6	Aug. 1	Mr. R. T.	23	62.7	22	116	4,380	$\frac{1}{14.3}$	6.99
Average.				65.4			3,888	$\frac{1}{16.8}$	5.95

It is hoped that the blood volume method with the easy technique for the carbon monoxide determination will be of more practical value than before.

SUMMARY.

Determinations of the blood volume by the carbon monoxide method with the simple technique previously described for the blood analysis have been made in fourteen rabbits and six normal men. Eleven determinations in seven male rabbits show an average blood volume of $\frac{1}{20.21}$ of the body weight, or 4.95 cc. per 100 gm. Nine determina-

tions in five non-pregnant female rabbits show an average of $\frac{1}{19.19}$, or 5.0 cc. per 100 gm.

In two pregnant rabbits the blood volume was largely increased, absolutely and relatively; about a week post partum it was restored to normal again.

The blood volume of rabbits may change from time to time, but the total oxygen capacity remains constant.

Seven determinations in six healthy men show an average of $\frac{1}{16.8}$ of the body weight, or 5.95 cc. per 100 gm.

The author wishes to express his thanks to Dr. Donald D. Van Slyke on whose initiative this work was undertaken, and to Mr. Arthur H. Smith for his technical assistance during the experiments.

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A STUDY BY THE SINGLE CELL METHOD OF THE INFLUENCE OF HOMOLOGOUS ANTIPNEUMOCOCCIC SERUM ON THE GROWTH RATE OF PNEUMOCOCCUS.

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Ascoli (1) attributed the immunity observed in certain experiments with anthrax to the antimetabolic action of immune serum on the bacilli, represented, in part, by the inhibition of capsule formation. He was the first to use the term antiblastic immunity in this connection. Dochez and Avery (2) found that the earlier growth of pneumococcus in homologous antipneumococcic serum was inhibited by the serum. They estimated the degree of inhibition by plating out samples and counting colonies. They reported a further inhibition of the metabolic activities of pneumococcus by homologous serum as measured by the production of amino-acids and by the fermentation of glucose, saccharose, lactose, and inulin. The serum of patients at different stages of pneumonia infection showed a like inhibitory action on the digestion of protein and the fermentation of inulin. Blake (3) repeated the experiments of Dochez and Avery and extended them. He obtained, in part, similar results, but put a different interpretation on the findings. Since pneumococcus tends to grow in chains and clumps in homologous serum, a sample of such growths would give fewer colonies than the controls, even when the numbers of bacteria were exactly the same. Further, the clumped masses of bacteria tend to collect in the bottom of the test-tube, and the lack of diffusion of formed products would explain the results of Dochez and Avery in regard to the fermentation of sugars and the production of amino-acids. In cultures frequently shaken or grown in shallow layers of medium in flasks Blake obtained the same metabolic activities in homologous serum as in controls. Homologous sera deprived of agglutinins gave the same results as heterologous sera. He confirmed the observation of von Dungern (4) that the liquefaction of gelatin by *Staphylococcus aureus* is inhibited by homologous serum, but, according to his observation this inhibition of liquefaction was not accompanied by a diminution of such metabolic activities as are essential to growth.

It seemed worth while to investigate further the possible inhibition of the growth of pneumococcus by homologous immune serum, with especial reference to the effect of the serum on the earlier genera-

tions, by a method which allows a comparatively accurate measurement of the growth rate of bacteria. The problem of infection and resistance is complex, involving factors the action of which is still obscure; and if one possible factor should be demonstrated and prove to be measurable, or if it should be definitely eliminated, we would have an advance in our knowledge of the mechanism of immunity.

In addition to accuracy in measurement of growth rate, the use of the single cell method offers other advantages in this problem. Similar sowings, the immediate progeny of a single cell, may be employed for the test culture and the controls. Agglutination may be eliminated, in the earlier generations, at least. Further, it is possible that the use of single or few cells in culture experiments may more closely simulate the process of infection in nature.

The method of single cell culture as described previously (5) was employed in these experiments with some improvements and minor modifications to suit the problem and the organism studied. No especial difficulty was encountered in working with pneumococci. They were easily isolated and grew readily, and multiplication in hanging drops proceeded apparently at the same rate as in the test-tube.

In practically all the experiments in hanging drops described in this paper, controls were made under the same conditions, so that it was not necessary to ascertain exactly the normal growth rate of pneumococcus. However, it was found that some approximation of this growth rate would be convenient, especially in estimating the degree of lag, when such occurred. Many tests showed that with Type I and Type II pneumococcus, grown in hanging drops under the best conditions—a favorable medium and temperature, and seeding material taken in an actively dividing stage—the growth rate approximates one generation in 30 minutes. In a few instances growth was apparently more rapid, possibly a generation every 25 minutes, but this occurred in only a small minority of the tests. Single pairs sown in test-tubes of 5 or 10 cc. of serum broth gave approximately the same rate. Here the number of cells in the culture was estimated by means of the counting chamber. It is hardly practicable to estimate the minimum generation time by means of plating out methods, since at the period of growth when the chains are breaking up, one

would get an apparent growth rate much more rapid than the real one. As the author has shown (6), colon bacilli also break up into much smaller elements at a certain period of growth, so that the plating out method with bacilli also is subject to the same error. In this paper the generation time of 30 minutes is taken provisionally as the normal rate for pneumococcus.

Experiments with Immune Horse Serum in Hanging Drops.

The method and results of hanging drop experiments are best illustrated by protocols of one or two experiments.

A single pair of Pneumococcus Type I, grown for some time on artificial media, was sown in a hanging drop of blood broth. After about five generations had formed, single pairs of the progeny of this pair were sown in hanging drops of homologous highly immune horse serum of Type I and also in drops of highly immune horse serum of Type II and of normal horse serum, all diluted 1:52 in nutrient broth. The growth was studied in five droplets of the diluted homologous serum, four of the heterologous serum, and two of the normal horse serum, all on the same large cover-glass and under like conditions. After 170 minutes incubation the cells in the droplets were killed and stained by injecting a stain through a capillary pipette under microscopic control. A special fixing and staining fluid, made by adding medicinal methylene blue to a solution of potassium hydroxide immediately before use, was employed. The number of pairs present was determined with a $\frac{1}{12}$ oil immersion lens, and the size of the organisms in the various droplets was compared and also compared with the size of the seeding pairs which had been noted at the time of inoculation.

In this experiment one pair in heterologous serum failed to grow. In all the remaining ten drops, including those containing the homologous serum, the amount of growth was almost exactly the same—slightly less than six generations—giving a growth rate of approximately 29 minutes. There was no greater tendency to lag in the homologous serum than in the controls.

In the experiment just described conditions were favorable and growth proceeded at a geometrical rate and at approximately the maximum rate for pneumococcus in hanging drops. In a second experiment the conditions were somewhat different. Undiluted serum was used with just enough nutrient agar added to make a thin jelly, two parts of agar to six of serum. In preparing the seeding material a single cell was isolated from a blood broth culture of Pneumococcus Type I, made from the blood of a pneumonia patient, and this cell was grown in a hanging drop of human blood broth to four generations. The cells formed adhered in a chain, and only four generations formed in 2½ hours, indicating that the pair sown was not actively growing at the time of isolation. This chain was broken up and distributed in drops of homologous and heterologous sera as shown in Table I.

TABLE I.

Growth of Pneumococcus Type I in Agar Serum Jelly. Incubation 180 Minutes.

Type I serum.			Type II serum		
Drop No.	Sowing. Pairs.	No. of generations formed.	Drop No.	Sowing. Pairs.	No. of generations formed.
1	1	4	1	1	4
2	1	3	2	2	4
3	3	3	3	3	3

In this experiment growth evidently lagged and the cells stained irregularly, so that the number of generations could only be approximated, but there was apparently no greater inhibition of growth in the homologous serum than in the control.

The protocols given above are representative of many hanging drop experiments. Sera were used undiluted and in dilutions up to 1:52. Dilutions were made in normal salt solution as well as in plain broth, glucose broth, and agar. Seeding material was taken from cultures long grown on artificial media, from infected human blood, and directly from the heart's blood of mice that had died of pneumococcus infection. In some experiments seeding material was taken at the height of growth, in others from a 22 hour culture, and in others from cultures at various other periods of growth. Lag and irregular growth occurred in some instances, especially when the seeding material was old or when it was sown in undiluted or little diluted sera, but in all these experiments the controls showed the same degree of irregularity and inhibition of growth as occurred in the homologous serum. To sum up, the hanging drop experiments gave no evidence of a measurable inhibition of growth in homologous serum.

While the growth rate in homologous serum was apparently unaffected, the character of the growth was distinctly different from that observed in heterologous serum or in other controls. The cells early became invested with a thick capsule and grew in chains, which often intertwined and formed zooglea-like masses. It was sometimes necessary to disentangle these chains by means of a fine capillary point, in order to count the cells. The capsule formed also on cells which were isolated from old cultures and were apparently dead or, at all

events, subsequently showed no growth in hanging drops. When sputum containing pneumococcus was incubated in a mixture of broth and homologous serum, these zooglea-like masses were distinctly seen, suggesting a possible means of early recognition of types of pneumococcus in sputum. This type of growth can hardly be called agglutination in the strict sense of the word, but resembles zooglea formation. The thick capsules are probably concerned in the protection of the organism, and their formation may be analogous to the formation of capsules by many bacteria in the animal body.

Experiments with Immune Horse Serum in Test-Tubes.

If homologous immune serum causes no inhibition of the growth of pneumococcus during the first five or six generations, it is not likely that any inhibition would occur in later growth, except that in the later periods the clumping of the cells and localization in one part of the container might interfere with the free diffusion of nutrient material and waste products, and in this way interfere with the free growth of the cells. Nevertheless, it seemed important to investigate this matter, and experiments of the following type were performed. One or more pairs of pneumococci were inoculated into test-tubes containing 2 to 10 cc. of diluted sera. In the experiment given in Table II 1 per cent glucose broth was used to dilute the serum, since the clumps formed in homologous immune serum when diluted with this medium apparently disintegrate earlier than when the dilutions are made in plain broth, and the cells can therefore be more readily counted. In this experiment homologous and heterologous antipneumococcic sera were employed in a dilution of 1:26 in 2 cc. of broth. As a control, Tube 7, which contained 11 cc. of glucose broth plus 0.1 cc. of rabbit serum, was included. As seeding material we used the progeny of one pair of Type I pneumococci, grown for 2 hours in a hanging drop of rabbit serum glucose broth. At the end of this period sixteen pairs had formed. The cells were apparently in an active state of multiplication.

The number of bacteria was ascertained by counting the stained cells in a counting chamber. In Tube 1 chains and clumps appeared, so that only an approximate count could be made, but, since even an

error of 100 per cent in counting would make a difference of only one generation, the error in the estimated generation time must be small. The growth in Tubes 3 and 4 was allowed to continue until the medium had become densely cloudy and the clumps had largely disintegrated. A more accurate count could then be obtained. In Tubes 3, 4, and 7, the growth had begun to lag on account of crowding before the count was made, so that the generation time is apparently greater in these tubes. All the cultures were well shaken at intervals after about 12 hours growth. Tube 2, containing Type II serum, showed cloudiness before any other. Otherwise the growth was ap-

TABLE II.

Growth of Pneumococcus Type I in Glucose Broth. Homologous and Heterologous Immune Sera, 1 : 26 Dilution.

Tube No.*	Type of serum.	Sowing. Pairs.	Length of incubation before cloudiness appeared.	Length of time allowed to grow before counting.	No. of generations formed.	Generation time.
			hrs.	*hrs.*		*min.*
1	I	1 medium sized.	$13\frac{3}{4}$	$14\frac{1}{3}$	26.2	32.8
2	II	1 " "	$12\frac{1}{4}$	$14\frac{1}{3}$	27.4	31.4
3	II	1 " "	13	$20\frac{1}{2}$	28.8	42.8
4	I	1 " "	13	$20\frac{1}{2}$	29.1	42.4
5	II	2 small.	No growth.			
6	I	2 "	" "			
7	Rabbit serum.	2 "	16	$18\frac{5}{6}$	30.2	34.3

* The tubes were inoculated in the order given.

proximately the same in all the tubes containing immune serum. In the two tubes containing homologous serum in which growth occurred, the clumping ordinarily observed in such serum was manifest.

In the following experiment (Table III) the homologous and heterologous sera were diluted 1: 10 in plain broth and 5 cc. quantities were used. The seeding material, Type I pneumococcus, was taken directly from a broth culture at the height of growth. The cells were isolated and each was separately transferred to the serum dilutions in the test-tubes. Six hanging drop cultures in plain broth were also made at the same time with cells isolated from the same source. In five of these cultures two generations occurred during

the 1st hour of incubation, and in one, one generation. It is probable therefore, that all cells sown into the serum dilutions were viable and actively growing.

On account of the formation of large clumps in the homologous serum tubes, it was hardly practicable to estimate comparative amounts of growth either by counting or by the degree of cloudiness. In consideration of the smallness of the sowings, it is apparent that growth in the homologous serum suffered little if any lag. The number of successful growths in the homologous serum was nearly as great as that in the heterologous, three out of five in the one and four out of five in the other.

TABLE III.

Growth of Pneumococcus Type I in Plain Broth. Homologous and Heterologous Sera, 1 : 10 Dilution.

Tube No.*	Type of serum.	Sowing. Pairs.	Growth after 15¼ hrs. incubation.
1	I	1	Clear above; clumps at bottom.
2	II	1	Cloudy ++.
3	I	1 small.	No growth.
4	II	1 "	" "
5	I	1 "	" "
6	II	1 "	Cloudy ++.
7	I	2 "	Clear above; clumps at bottom.
8	II	2 "	Cloudy ++.
9	I	4 "	Clear above; clumps at bottom.
10	II	4 "	Cloudy ++.

* The tubes were inoculated in the order given.

In another experiment 10 cc. lots of broth plus homologous serum, diluted 1: 50 and 1: 100, were employed, and the growth in these was compared with the growth in similar quantities of the same broth without any serum. Sowings of one and two pairs were made. There was less lag in the tubes containing serum than in the plain broth tubes, probably entirely due to the presence of the serum. Clumping occurred in the 1: 100 dilution but in less degree than in the 1: 50 dilution. Growth was visible macroscopically in both dilutions at the end of 15 hours, while the plain broth tubes were still clear 3½ hours later.

In short, the test-tube experiments show that growth in homologous serum proceeds at nearly, if not quite the same rate as that in the heterologous serum in spite of the formation of clumps.

Action of Homologous Serum Plus Complement.

In the experiments described above no fresh complement was added other than that contained in blood or serum broth. In order to test the effect of homologous serum plus complement on growth rate, preliminary experiments were arranged in which varying amounts of fresh rabbit blood, fresh human blood, or rabbit blister fluid containing more or less active leucocytes were added to homologous serum. In these mixtures the homologous serum gave no indication of a greater power to restrain growth of pneumococcus than the controls.

Experiments in Vivo.

Intravenous Injection of Immune Serum.—It was then proposed to ascertain whether homologous serum acquired some growth-inhibiting substance through contact with animal tissues *in vivo*.

A normal rabbit, weighing 3,200 gm., was given intravenously 5 cc. of a Type I immune horse serum. 1 hour later the rabbit was bled from the ear. Before coagulation had taken place, part of the blood was diluted in plain broth, one part of blood to four of broth, and another portion was collected in a centrifuge tube, in order to obtain serum for the tests to be described later. To serve as controls, blood of the same rabbit taken immediately before the injection of the serum was similarly diluted, and, in addition, a second control was made by adding 0.5 cc. of the normal rabbit blood to 9 cc. of broth containing 0.5 cc. of the same Type I serum that was inoculated into the rabbit. The tubes were immediately transferred to the ice chest. A series of hanging drop cultures was made at the same time in the 1:5 dilutions of normal blood, in the blood taken after the serum injection, and in the mixture containing normal blood, immune serum, and broth. The inoculation of these drops was made with cells of Pneumococcus Type I taken from a young broth culture. After 2 hours incubation the growth formed was stained on the coverglass and the cells were counted under the $\frac{1}{12}$ oil immersion lens. The results are given in Table IV.

TABLE IV.

Growth of Pneumococcus Type I in Hanging Drops. Normal and Immune Rabbit Blood, Dilution 1:5 in Broth. Incubation 2 Hours.

Normal blood.			Blood after injection of immune horse serum.			Normal blood plus homologous immune horse serum.		
Drop No.	Sowing. Pairs.	No. of generations formed.	Drop No.	Sowing. Pairs.	No. of generations formed.	Drop No.	Sowing. Pairs.	No. of generations formed.
1	1½	0	1	1	3	1	1	3
2	1	4−	2	2	3	2	3	3
3	2	4	3	2	3+	3	1	3+
4	2	3−	4	1½	0	4	2	3+
5	1	4+	5	1½	0	5	1	3+
6	3½	2+	6	1	1	6	1	3
7	2	3	7	1	4	7	1	2
8	1	3	8	1	1	8	½	0
9			9	1	3	9	2	3

It is seen in Table IV that growth took place in a large proportion of the hanging drops, averaging about three generations in 2 hours. A varying amount of lag is shown, due possibly to unevenness in the sowing material, but, in the aggregate, growth was practically the same, in all the groups. The tendency to capsule formation, characteristic of growth in homologous serum, appeared in all except the controls in normal blood. The experiment was repeated on the same day, with actively dividing cells of like ancestry grown on the cover-glass as sowing material. The same 1:5 dilutions in broth of blood taken before and after immunization were employed, but plain broth was substituted for the control with normal blood plus homologous serum. Growth proceeded at nearly the normal rate for hanging drop cultures —three to four generations in 90 minutes. The rate of growth in the blood of the animal which had received immune serum was practically the same as that in the blood of the normal animal.

On the following day a comparison was made of the undiluted serum of the rabbit taken before and that taken 1 hour after injection of immune serum. Seven hanging drops were made of each, and sowings were made from an actively growing broth culture. The serum was undiluted except through the small amount of moisture of conden-

sation present on the cover-glass. Plain broth controls were used. Growth occurred in all, with the exception of one broth control, but tended to lag in the undiluted serum. There was no measurable difference between the number of generations formed in the normal serum and in that obtained following the injection.

In order to compare the results obtained in hanging drops with those obtained in test-tubes an experiment was arranged with the same sera that were used in the last experiment. The sera were diluted in plain broth, 0.5 cc. of serum to 4.5 cc. of broth. Sowings were taken from a young plain broth Pneumococcus Type I culture, and, in order to get similar cells, one long chain of cocci was broken up to furnish the sowings. The results are given in Table V.

TABLE V.

Growth of Pneumococcus Type I in Test-Tubes. Normal and Immunized Sera, Dilution 1:10 in Broth.

Tube No.*	Rabbit serum.	Sowing. Pairs.	Growth after 15 hrs. incubation.	Growth after 19½ hrs. incubation.
1	After injection of immune serum.	2	Clear above with a clumped mass in the bottom.	Cloudy ++.
2	Before injection of immune serum.	4	Cloudy ++.	" ++.
3	After injection of immune serum.	2	Clear.	" ++.
4	Before injection of immune serum.	2	Cloudy ++.	" ++.

* The tubes were inoculated in the order given.

It will be noted in Table V that growth took place in all the tubes, with some lag in No. 3. However, in consideration of the variability of lag in test-tube experiments of this kind, whether immune serum is present or not, the lag in this tube can hardly be attributed to the presence of the homologous serum.

The experiments with the serum of the passively immunized rabbit were repeated with the serum of another similarly immunized animal, with especial attention to any inhibitory substance in the whole blood or undiluted serum. A 3,000 gm. rabbit was given intravenously 5 cc. of a potent Type I serum. 23 hours later a sample of blood was

drawn from an ear vein into a capillary pipette, and, before coagulation had taken place, was added to sowings previously isolated in minimum sized drops on the cover-glass. The sowings were taken from an actively growing rabbit blood broth culture of Type I pneumococcus. To serve as controls, a similar series of drop cultures was prepared on the same cover-glass with normal rabbit blood and rabbit blood broth. The results after $2\frac{1}{2}$ hours incubation are given in Table VI.

TABLE VI.

Pneumococcus Type I. Culture in Immunized Rabbit Blood, in Normal Rabbit Blood, and in Broth.

Blood of passively immunized rabbit.			Blood of untreated rabbit.			Broth.		
Drop No.	Sowing. Pairs.	No. of generations formed.	Drop No.	Sowing. Pairs.	No. of generations formed.	Drop No.	Sowing. Pairs.	No. of generations formed.
1	$\frac{1}{2}$	5	1	1 small.	4+	1	$\frac{1}{2}$	5
2	$\frac{1}{2}$	5	2	1 medium sized.	4+	2	$\frac{1}{2}$	5
3	1 large.	5	3	" "	4+	3	$\frac{1}{2}$	5
4	$\frac{1}{2}$	5	4	" "	4+	4	$\frac{1}{2}$	0
5	1 large.	5+	5	$\frac{1}{2}$	5	5	$\frac{1}{2}$	5
6	$\frac{1}{2}$	5	6	2 large.	5	6	1 small.	5±
7	2 medium sized.	5+	7	2 medium sized.	4			

As shown in Table VI all the droplets showed growth except one of the broth, and growth occurred in all at approximately the normal rate. The cells in the immune blood formed chains characteristic of growth in homologous serum, but showed no lag as compared with the controls.

Similar series of cultures were made in the whole blood of the same rabbit taken $1\frac{1}{2}$ and 21 hours after the injection of serum. In neither series was there any evidence of inhibition of growth attributable to immune substances in the whole blood.

A series of test-tube experiments was then done with the whole blood, the serum, and the plasma of the immunized rabbit taken 3 days after the injection of the immune serum. Sowings of cells taken from an

actively growing Type I broth culture were made into the bottom of dry test-tubes. Into each tube only a single chain was sown, the chains varying in length from four to seven pairs each. Only sufficient broth to expel the chains from the pipette, amounting in each instance to a single small droplet, was discharged into the tubes. Three of the tubes so prepared received 1 cc. each of whole uncoagulated blood, not citrated; three received 2 cc. each of fresh serum; three, 2 cc. of fresh plasma; and three, 2 cc. of rabbit blood broth to serve as controls. The next day growth was evident in all except the three which received the whole blood. The growth appeared in the coagulated plasma in the form of single colonies in two of the tubes and as two colonies in the third. The growth in the serum and plasma was plated out and proved to be pneumococcus. The failure of growth in the whole blood might be attributed to phagocytosis, judging from the positive results obtained in hanging drops, but of this there is no definite proof.

On the same day the growth in plasma, in undiluted serum, and in whole blood from the same rabbit was compared with the growth in rabbit blood broth in hanging drops in much the same way as in the experiment described in Table VI. Growth failed in one of seven plasma droplets and in three of seven whole blood droplets. In the whole blood there was some evidence of phagocytosis in three of the droplets in which growth failed or was deficient. Growth occurred in all the serum droplets and in the broth controls. In all four sets when positive results were obtained, the amount of growth was approximately the same.

In summarizing all the experiments done with homologous serum, whole blood, or rabbit blood plasma *in vivo*, we find no measurable influence of the immune serum on the growth rate of pneumococcus.

Action of Homologous Serum in Vivo, in the Peritoneum of the Mouse.

In a series of experiments a study was made of the behavior of pneumococcus in the peritoneal cavity of normal and immunized mice. Mice were passively immunized by means of the injection of measured amounts of immune serum at varying periods prior to the injection of the bacteria. Sometimes the same mouse was used repeat-

edly for different experiments, so that it may have acquired some degree of active immunization by virtue of the repeated doses of bacteria. Cultures of different degrees of virulence and at different stages of growth were compared, and various sized doses of cocci were given. The object of the experiments was to ascertain whether the disappearance of the pneumococcus in the peritoneal cavity of the immunized mouse is due in any measure to the humoral activity of the peritoneal fluid. The protocols of several experiments will illustrate the method and results.

A mouse was given intraperitoneally 0.2 cc. of Type I antipneumococcic serum, and 1 hour later received 0.1 cc. of a rabbit blood broth culture of Type I pneumococcus, also intraperitoneally. The culture was the second transfer from the heart's blood of a mouse and had grown $15\frac{1}{2}$ hours at high room temperature. It was very cloudy and contained mostly discrete pairs or short chains. A test of viability was made at the time of inoculation by sowing single pairs of cells into hanging drops of broth. Twenty-six out of twenty-seven of these pairs grew, but the early growth was at only about half the normal rate. So practically all the cells inoculated were viable but not at the full height of growth.

At the same time that the immunized mouse was inoculated, a normal control received exactly the same dose of the same culture.

Samples of peritoneal fluid were taken from both mice by means of capillary pipettes 1 hour and 10 minutes, 4 hours, and 6 hours and 10 minutes after the inoculation of the bacteria. Additional samples were taken from the immunized mouse at about 8 hours (7 hours and 55 minutes) and at 28 hours after inoculation. In the control mouse numerous leucocytes and numerous cocci, mostly in separate pairs, were found at all periods. Phagocytosis, if present at all, was little marked. The mouse died during the night following the inoculation of pneumococci. In the immunized mouse many leucocytes and a few extracellular cocci, mostly in short chains, were found at the end of the 1 hour and 10 minute period. Some of these free chains were isolated into hanging drops of peritoneal fluid and grew at apparently the normal rate. Phagocytosis was marked, and the number of cocci found in phagocytes was apparently sufficient to account for the diminution of free cocci in the peritoneal fluid. At the end of the 4 hour

period and thereafter no extracellular bacteria could be found, although they were distinctly demonstrable in leucocytes at the end of 8 hours.

It was demonstrated that cocci contained within leucocytes of the immunized mouse were viable as late as 6 hours after inoculation and 2 hours after the period when extracellular cocci had apparently disappeared. This was proved by the following experiment.

Hanging drops made of the peritoneal fluid both undiluted and diluted in broth were allowed to incubate, and were then stained by means of a capillary pipette under microscopic control. These preparations were compared with similar preparations made from drops which were stained before incubation. In some leucocytes there was apparently no growth of cocci during incubation and the bacteria in some instances appeared to have degenerated. In others, small colonies had formed in and around the phagocyte. No evidence of bacteriolysis was found which could be ascribed to the effect of the homologous serum. A few free bacteria stained irregularly, but in no larger proportion than may be found in normal serum.

Hanging drop and plate cultures of peritoneal fluid were in all cases compared with films on slides subsequently stained by Gram's method and with Manson's methylene blue. The immunized mouse remained alive and well 6 days after the experiment.

In another experiment extracellular cocci taken from the peritoneal fluid of an immunized mouse were isolated in hanging drops containing a mixture of peritoneal fluid and broth. These cultures showed growth at fully the normal rate, five generations in $2\frac{3}{4}$ hours. In this case the mouse had received 0.2 cc. of homologous serum, followed by 0.05 cc. of a culture of Pneumococcus Type I, the first transfer from the heart's blood of a mouse. Tests in hanging drops had shown that practically all the bacteria inoculated were viable. This mouse had received no injection of bacteria or serum previous to the day of the experiment. The peritoneal fluid for the test was removed 35 minutes after injection of the bacteria. Phagocytosis was marked. After 1 hour and 25 minutes the fluid contained no free bacteria.

Growth Rate in Vivo in the Peritoneal Fluid of the Passively Immunized Mouse.

As noted in the previous experiments non-phagocytosed pneumococci isolated from the peritoneal cavity of the mouse grow in hanging drops at the normal rate. It was next proposed to ascertain whether growth takes place *in vivo* in the peritoneal fluid and to attempt to determine approximately the rate of growth, advantage being taken of the tendency of Pneumococci Type I to adhere in chains in homologous serum. These experiments were suggested by the observation that extracellular pneumococci found after 1 or 2 hours in the peritoneal cavity were usually in chains. One protocol will illustrate the method and results.

A mouse was given intraperitoneally 0.2 cc. of Type I serum. About $1\frac{1}{2}$ hours later it was given a dose of Type I culture. The inoculum was prepared as follows: A rabbit blood broth culture was incubated until it became well clouded and examination of hanging drops showed that most of the chains had broken up. A portion of the culture was then centrifuged at low speed for about 5 minutes, in order to throw down the chains. The supernatant fluid was nearly clear and hanging drop examination showed that no long chains remained. A series of cells was isolated in hanging drops and all proved viable and actively dividing. 0.4 cc. of the supernatant fluid was inoculated intraperitoneally. A sample of peritoneal fluid was taken in about 2 minutes, a second sample 60 minutes later, and further samples at 30 minute intervals. The samples were placed on slides with little spreading, dried, fixed in alcohol, and stained with Manson's methylene blue. The incidence of pneumococci in pairs or in chains of various lengths was noted in several fields taken at random. The results of the countings are given in Table VII.

Samples taken at 150 minutes and later showed nearly all the chains either in phagocytes or broken into irregular groups. Up to the fourth sample the chains were fairly intact, owing to the capsular material which held them together. A few chains in the fourth sample had begun to break up. Phagocytosis became marked at 90 minutes, bacteria became fewer, and an increasing proportion of the contents of the phagocytes consisted of longer chains. Few single pairs were found at the end of 60 minutes. The mouse recovered.

By comparing the incidence of chains in the different samples in Table VII it becomes evident that growth was going on at a rate of about one generation in 30 minutes. In the first sample most of the cocci were in single pairs, with the next greatest number in chains of two pairs. 60 minutes later the greatest number of cocci were in chains of 4 and 4 to 8 pairs. 30 minutes later the largest number of cocci were in chains of 8 and 8 to 16 pairs. This indicates that the rate of division in the peritoneal cavity approximates the usual rate observed in hanging drop cultures.

TABLE VII.

Length of Chains of Pneumococci in Samples of Fluid Recovered from the Mouse Peritoneum.

Sample No.	Length of time after inoculation.	No. of pairs of cocci per chain.									
		1	2	2–4*	4	4–8	8	8–16	16	16–32	32
	min.										
1	2	94	41		4						
2	60	1	2	3	31	10	5	1			
3	90					6	25	17	7	1	
4	120	†					3	7	23	11	9

* The headings 2–4, 4–8, 8–16, 16–32 refer to chains some members of which were dividing to form chains of a higher order.

† Some single pairs occur in clusters, probably from broken chains. Three 3 pair chains, four 6 pair, and six irregular chains are not included.

A parallel experiment with Type I pneumococcus was done on a mouse which had received during the 10 days previous to the experiment three doses approximating 0.05 to 0.1 cc. each of broth culture of pneumococcus together with homologous serum. These inoculations had been made in connection with other experiments and some active immunity may have been conferred on the mouse. This mouse was passively immunized on the day of the experiment and almost exactly the same procedure was followed as in the experiment described in Table VII. The results were similar except that in this case the chains held intact longer and could be counted at the end of 205 minutes, when chains of 64 to 128 could be found. About the same growth rate was observed as in the previous experiment. This mouse also recovered.

Similar experiments were also done with Pneumococcus Type II. This type proved less favorable, since the chains lost their identity earlier and tended to fuse into masses, owing to the more abundant formation of capsular material. In one experiment, however, three samples taken at about 30 minute intervals gave similar results to those of the Type I experiments. In this experiment only 0.2 cc. of supernatant culture fluid was given. Phagocytosis began in about 30 minutes and continued actively. However, this mouse died 4 days after inoculation with pneumococcus.

Apparently the growth of extracellular pneumococci in the peritoneal cavity of the immunized mouse continues until the chains or groups are overtaken by phagocytosis, and the growth of some portion, at least, of the cells continues at a geometrical rate. A few chains were noted in which a part of the members failed to take the stain deeply. This can hardly be considered as due to the action of the immune serum, since a similar phenomenon is sometimes observed in chains formed in undiluted normal blood or serum. At all events, the proportion of such degenerated cells in the mouse peritoneum is too small for us to ascribe much importance to this phenomenon as a factor in the disappearance of the bacteria.

Capsule formation was marked in the peritoneal cavity of the immunized mouse, but in Type I pneumococcus infection this apparently interfered little with phagocytosis. In Type II infection groups of bacteria formed thick gelatinous masses, which may have offered some mechanical hindrance to the phagocytes, but there is no proof in these experiments that these capsules were an important element in the protection of the bacteria.

DISCUSSION.

The studies which have been described, in which the single cell method of cultivating bacteria was employed, have not been successful in demonstrating that immune antipneumococcic serum owes any of its effect to any power of inhibiting the growth of pneumococci. While it is still possible that the serum may have such an effect, it has not been possible to demonstrate it by the method employed. That antipneumococcic serum has a high protective and curative

value has been demonstrated, but the mechanism of this action is still not entirely clear. The attempts to analyze its mode of action have so far only succeeded in showing that it has the power of causing marked agglutination of homologous organisms and of changing the organisms so that they undergo phagocytosis. The work of Bull (7, 8) shows that the agglutination of bacteria *in vivo* is probably a more important phenomenon in immunity and recovery than was formerly believed. The fact that the protective power of any lot of antipneumococcic serum, as tested *in vitro*, is not always proportional to its agglutinating power is opposed to the assumption that agglutination is of paramount significance. The exact importance of the property of facilitating phagocytosis has also not been thoroughly determined. The fact that the only real test of antipneumococcic serum is its action *in vivo*, in protecting the life of infected animals, seems to indicate that its effect depends, in part at least, on properties which are only manifested *in vivo* and the nature of which are not yet fully understood.

CONCLUSIONS.

1. Under the conditions stated pneumococci grow as readily in the serum of horses highly immunized to the homologous organism as they do in normal horse serum, and the rate of growth is not appreciably diminished.

2. This failure of immune serum to affect the growth rate is not altered when fresh rabbit blood, fresh human blood, or rabbit blister fluid is added in order to supply any hypothetical complement which might be lacking.

3. We have not been able to show that when immune horse serum is injected intravenously into rabbits or intraperitoneally into mice, it acquires the property of killing pneumococci or inhibiting their growth.

4. Experimental evidence has been obtained indicating that in the peritoneal cavity of the passively immunized mouse the growth of extracellular pneumococci continues at apparently the normal rate, until the bacteria are engulfed by phagocytes.

5. The immunizing and protective power of antipneumococcic serum probably depends, in part at least, on properties which are not

at present known. It has not been possible in the present study to demonstrate that one of these properties consists in delaying the growth of pneumococci.

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ANTIBLASTIC PHENOMENA IN ACTIVE ACQUIRED IMMUNITY AND IN NATURAL IMMUNITY TO PNEUMOCOCCUS.

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In the preceding paper (1) the results are reported of an investigation of the power of the serum of highly immunized horses to inhibit the growth rate of pneumococci. In that investigation, in which the so called single cell method of cultivating organisms was employed, it was impossible to demonstrate any growth-inhibiting action of the serum. This was true even when the serum was placed in the peritoneal cavity of the mouse and under conditions in which its full protective action was being manifested. Nevertheless, it seemed important to exclude the possibility that while no inhibiting action by serum can be demonstrated, the whole blood when freshly drawn from the actively immunized animal may have an effect of this kind. It seemed possible that the so called antiblastic immunity might play a part in active immunity, although the serum of the actively immunized animals might exert its effect in producing passive immunity in an entirely different manner. Consequently, a series of experiments was undertaken to determine whether or not blood or plasma fresh from the immune horse had any effect on the growth rate of pneumococcus. The same single cell method of study employed in the previous investigation was used in this work.

A virulent strain of Pneumococcus Type II was used in all the tests, and the blood was furnished by a horse which had received many injections of the same strain and of various atypical Type II strains as well. The degree of immunity was such that 0.2 cc. of serum protected mice against 10,000 lethal doses of the Type II strain employed in these experiments.

Tests with Whole Fresh Blood and Fresh Serum Diluted in Broth.

In the first series of experiments broth dilutions of whole blood and fresh serum were compared with fresh normal horse serum controls. Both hanging drop and test-tube series were done, the latter with sowings of chains of one to three pairs into quantities of medium varying from 4 to 10 cc. The whole blood was diluted 1:11 and the serum 1:5. In all the tests growth occurred in the dilutions of the immune blood as well as in the controls. The growth rate, as shown by growth in hanging drops, was practically the same in the immune blood and serum as in the controls.

Tests with Whole Citrated Blood.

The citrated blood was used on the day of collection, with normal horse serum as a control, both undiluted. The normal horse serum had been kept some months in the ice chest. The results after 2 hours incubation are given in Table I.

TABLE I.

Growth in Hanging Drops of Whole, Undiluted Citrated Blood of an Actively Immunized Horse Compared with That in Hanging Drops of Undiluted Normal Horse Serum.

Pneumococcus Type II.

Immune citrated blood.			Normal horse serum.		
Drop No.	Sowing. Pairs.	No. of generations formed after 2 hrs.	Drop No.	Sowing. Pairs.	No. of generations formed after 2 hrs.
1	2 small.	4	1	1 medium sized.	3+
2	2 "	4	2	2 small.	3+
3	1 medium sized.	4+	3	1 large.	4−
4	2 " "	4+	4	1 medium sized.	4−
			5	1 large.	4

There was therefore no evidence in these experiments that the whole fresh citrated blood from the immunized horse caused any delay in the growth rate of homologous pneumococci. Further experiments were made with fresh citrated blood in test-tubes, diluted and undiluted, but no definite evidence of inhibitory action could be obtained.

It was then determined to test the effect of whole undiluted blood transferred directly from the ear of the horse to sowings of one and two pairs of pneumococci previously isolated on the cover-glass, the method previously described being followed. Fresh normal horse blood was used as a control. After 3 hours incubation the greatest amount of growth in any droplet was two generations, and few showed that much. The amount of growth in the blood of the immunized horse and in the control was about the same.

Many hanging drop experiments were also done with the fresh undiluted coagulated plasma of both the normal and the immunized horse. In obtaining plasma for these experiments, blood contained in centrifuge tubes was placed on ice immediately after draining from the animal. The uncoagulated blood was centrifuged free from cells and kept cold until ready for use. The plasma coagulated soon after exposure to higher temperatures, but remained fluid long enough to allow mixing with organisms previously isolated in test-tubes or on the cover-glass. In every case without exception growth either failed or was limited to one generation after $2\frac{1}{2}$ hours of incubation. There was rarely, if ever, any trace of bacteriolysis, however, and the organisms sown could usually be found after incubation. Broth controls of the seeding material grew well. The effect of coagulated plasma was also tested by mixing it in amounts of 2 cc. in the bottom of test-tubes with minute droplets of broth containing six to eight pairs of pneumococci. Growth almost always occurred both in the tubes containing plasma from the immunized horse and in those containing plasma from the normal horse. There was some evidence that growth under these conditions tends to lag, however, but this effect was observed both in the plasma of the immune and in that of the normal horses. Similar experiments were done with the fresh undiluted serum of the same normal and immune horses. Here too it was found that in hanging drops a marked prolongation of generation time occurred which was evident both in the normal and in the immune serum.

These experiments indicate that under the conditions described whole fresh horse blood and coagulated plasma, and also serum, are not favorable media for the growth of pneumococci. They do not

indicate, however, that this fact is at all related to the phenomena of immunity, since it is exhibited in both immune and normal horse blood.

Natural Immunity.

While the previous experiments have not afforded any evidence that inhibition of growth of pneumococci by serum or body fluids plays any part in acquired immunity, it is possible that natural immunity against pneumococcus infection may depend to some extent upon this property of the serum. The inhibiting effect of whole horse blood upon the growth of pneumococci might give some support to this view. The relative susceptibility of the horse to pneumococcus infection is not accurately known, but it is not generally considered to be very high as compared with that of the mouse or rabbit, for instance. Certain experiments previously performed (1), while not planned with this point in mind, give strong evidence that whole rabbit blood, unlike horse blood, does not have an inhibiting effect upon the growth of pneumococci.

Heist, Solis-Cohen, and Solis-Cohen (2) have reported that by a special method which they have devised, they have demonstrated a bactericidal action in the blood of chickens and pigeons, animals which are known to be highly immune to pneumococcus infection. Strouse (3), who studied the question of natural immunity of the pigeon, was unable to demonstrate any bactericidal action of pigeon serum and presented evidence to show that this immunity was due to the normal high temperature of the pigeon which was inimical to the growth of pneumococci. Kyes (4) also found no evidence of pneumococcidal action of pigeon serum *in vitro*.

Heist, Solis-Cohen, and Solis-Cohen studied fresh whole pigeon blood. They used small glass pipettes, and sowings consisted of the small numbers of bacteria which adhered to the walls after the pipettes had been filled and emptied from broth cultures of pneumococci. The fresh blood is then brought directly into contact with these bacteria by sucking the blood into the tube. The tubes are sealed off, incubated, and the contents later blown on glass slides and stained. It is stated that by this method, with not only the undiluted broth cultures, but dilutions of the broth cultures as well for seeding, it is possible to ascertain whether bactericidal action has occurred and to what degree. They state that with this method they have demonstrated that pneumococci fail to multiply in pigeon blood. Chicken blood is also bactericidal but less so than pigeon blood, while normal rabbit blood and mouse blood have no such action. Immune rabbit

blood, however, has been shown in this way to have a specific bactericidal action, and they state that they have also demonstrated this action in the blood of recovered patients.

The method employed by these writers is, in our opinion, open to certain objections, and it seemed important to investigate this matter further, since it has a direct bearing on our problem.

In our experiments the hanging drop method with small sowings was employed. A minimum of broth was used in the isolation of the organisms, in some instances scarcely more than that of the volume of several erythrocytes. A record kept of the relative amount of broth employed showed that there was apparently no greater amount of

TABLE II.

Growth in Hanging Drops of Fresh Citrated Pigeon Blood Compared with That in Hanging Drops of Rabbit Blood.

Pneumococcus Type I.

Rabbit blood.			Pigeon blood.		
Drop No.	Sowing. Pairs.	No. of generations formed.	Drop No.	Sowing. Pairs.	No. of generations formed.
1	1 large.	3+	1	1 large.	2
2	1 "	3+	2	1 "	2−
3	1 "	3±	3	1 "	2−
4	1 "	3	4	1 "	2−
5	1 "	3+			

growth in the drops which were intentionally given a slightly larger amount of broth. After the isolations had been made, a relatively large drop of blood was added to each seeding in such a way that the blood was caused to engulf the minute droplet containing the isolated bacteria. By this method the behavior of an exactly known sowing could be followed under conditions in which any humoral factor might act, but in which there was a minimal chance of any phagocytosis occurring without being observed.

In two series of experiments fresh citrated undiluted pigeon blood was used. A protocol is given in Table II. A Type I pneumococcus culture, apparently somewhat past the maximum height of growth, was used for seeding. The time of incubation was 2 hours. Growth

occurred in all the droplets in both samples with a tendency to lag in both. The growth in the pigeon blood was about one generation behind that in the rabbit blood. The cocci tended to form chains in the pigeon blood and discrete elements in the rabbit blood.

A second experiment with virulent Type II culture gave essentially the same results.

In another experiment non-citrated blood taken from another pigeon was used. The organisms were isolated in a minimum of broth as before. Fresh non-citrated rabbit blood was used as a control. The animals were brought to the microscope and the blood was transferred by means of a capillary pipette to the droplets before coagulation had occurred. In one series the droplets were incubated for 5 hours in order to ascertain whether growth in the blood would continue beyond the first three or four generations. Actively dividing Type I cells taken from a broth culture were used for the sowings, which consisted of one pair or of a chain of two very small pairs per droplet. Of five droplets of rabbit blood all showed growth and gave approximately nine generations each. Of five droplets of pigeon blood, four showed growth, with approximately seven, six, two, and six generations respectively, a lagging of two to seven generations behind the growth in rabbit blood. The cocci in the rabbit blood tended to form groups of discrete elements, while those in the pigeon blood were mostly in chains.

Other experiments were made with the non-citrated fresh pigeon blood, and in these practically no difference appeared between the growth in rabbit and pigeon blood. A protocol of one of these is given in Table III. Sowings were taken from an actively dividing Type I culture, the same strain that was used in the previous experiments, and the time of incubation of the droplets was $2\frac{1}{2}$ hours.

Here the amount of growth was essentially the same in both blood samples, and in most droplets multiplication went on at the normal rate. In the pigeon blood there was a greater tendency to chain formation, and in the two droplets in which growth was deficient there was some evidence of phagocytosis.

In summarizing these experiments we may say that growth occurred about as often in the pigeon as in the rabbit blood, and the rate of growth in both was practically the same. By the method employed by

us, therefore, we were unable to demonstrate that pigeon blood has any bactericidal action or inhibiting effect on the growth of pneumococci. It is possible that the differences between our results and those of Heist, Solis-Cohen, and Solis-Cohen are due to the differences in the conditions of the experiment. However this may be, we have, by the technique employed, been unable to show any relation between natural immunity in the pigeon and inhibition of growth of pneumococci by the whole blood.

TABLE III.

Growth in Hanging Drops of Non-Citrated Pigeon Blood Compared with That in Hanging Drops of Rabbit Blood.

Pneumococcus Type I.

Rabbit blood.			Pigeon blood.		
Drop No.	Sowing. Pairs.	No. of generations formed.	Drop No.	Sowing. Pairs.	No. of generations formed.
1	½	5	1	½	4+ or 5
2	½	5	2	1½	2±
3	½	0	3	½	5
4	1 small.	5±	4	1 large.	5
5	½	4+	5	½	5
6	½	4+ or 5	6	1 small.	5
			7	1 "	3 or 4

SUMMARY AND CONCLUSIONS.

The paper gives the results of a series of experiments made with a special technique for the purpose of testing whether or not so called antiblastic phenomena or bactericidal phenomena play any part in immunity to pneumococcus. The technique employed is that devised by the writer and consists in the isolation of single bacteria and their growth in hanging drops where growth can be continuously observed. From these experiments it may be concluded that: (1) Whole fresh blood, coagulated plasma, or serum of the immunized horse added directly to pneumococci, has considerable inhibiting action on the growth of pneumococci. The inhibiting effect of the fresh blood, coagulated plasma, or serum of a normal horse, however, is as marked as that of a horse highly immunized to pneumococci of the same type.

This property, therefore, does not seem to be of importance in acquired immunity in the horse. No such property has been demonstrated in whole fresh rabbit blood. (2) It has been impossible to demonstrate that antiblastic phenomena play any part in natural immunity to pneumococcus, at least as far as the immunity of the pigeon is concerned. The whole fresh blood of the pigeon under the conditions employed exhibits no inhibiting action on the growth rate of pneumococci.

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SOME MORPHOLOGICAL AND BIOLOGICAL CHARACTERS OF THE SPIRILLA (VIBRIO FETUS, N. SP.) ASSOCIATED WITH DISEASE OF THE FETAL MEMBRANES IN CATTLE.

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Plate 16.

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In an earlier communication[1] a spirillum of definite morphological and cultural characters was described as being associated in a series of cases with what is commonly known as infectious abortion in cattle. In the following pages the statements there made concerning the biology of the spirillum are amplified and supplemented by fresh observations and by studies on the agglutinative affinities of the various strains. In all, 24 fetal strains have been kept under cultivation. Of these one (No. 308) is a slightly modified, aberrant type. All were obtained from one large herd into which cattle from outside are introduced at irregular intervals.

Morphological Characters.

In films and in hanging drop preparations from fetal fluids and cultures therefrom, the spirilla appear as fine wavy or sinuous lines of various lengths. The smallest forms appear as minute curved S-shaped lines; the longest may stretch nearly across the field of the microscope. If we assume that the spirillum is in the form of a spiral or corkscrew, the diameter of the spiral is small. The spiral is drawn out, as it were, becoming in some cultures almost a straight line. In dried and stained films the spiral becomes a shallow sinuous line (Fig. 7). No segmentations are distinguishable in the longer forms.

[1] Smith, T., *J. Exp. Med.*, 1918, xxviii, 701.

As regards size, the width of the spirillum stained in alkaline methylene blue is probably not over 0.2 to 0.3μ, the shortest form about 1.5 to 2μ long. A common size in the fetal fluids consists of about two complete turns and measures 4 to 5μ in length. As stated above, the diameter of the spiral or turn varies somewhat, but it averages about 0.5μ.

The organism stains fairly well in alkaline methylene blue provided the staining is prolonged, preferably over night. It stains much more deeply in diluted aniline water gentian violet (Fig. 6), but the former stain is to be preferred since it requires no decoloration, a process likely to decolorize the spirillum itself.

When Löffler's flagellar stain, as modified by Moore,[2] is applied, the organism is much thicker, as shown in Figs. 1 to 5. A flagellum is found attached to one or both poles of the spirillum. Actual counts of the spirilla of eight different strains in a given number of fields indicated that organisms having unipolar flagella are far more numerous than those with bipolar flagella. The latter may be considered relatively scarce. The unipolar forms may be individuals recently set free by division, the bipolar forms ready to divide. Longer spirilla have been seen with several short lateral flagella in addition to the terminal ones. This appearance may be due to forms which have divided but not yet separated, the flagella of the daughter cells having been formed in the meantime. The character of the flagella is best seen in the figures. They are very fine hair-like processes in large undulations or waves, of uniform width throughout.

In hanging drop preparations the shorter forms, from $\frac{1}{2}$ to 2 windings long, are very active. They shoot with lightning rapidity in straight lines across the field in all directions. Only occasionally does one reduce its velocity so that details of the movements become visible. It may then be seen in a few cases that the organism revolves around its longitudinal axis. Whether this motion is used in very rapid propulsion cannot be determined. The longer forms move sluggishly or are quiescent.

Another element brought out by staining and referred to in a former paper[1] is the granules which appear on or within the spirilla. They

[2] Moore, V. A., Wilder quarter century book, Ithaca, 1893, 339.

occur more commonly in old cultures and are probably degeneration forms. Usually the long spirilla contain many, but short forms are not without them. In the latter they are terminal. In the long forms they are arranged along the filament at fairly regular intervals. They stain much more deeply than the rest of the spirillum and suggest the Neisser granules of diphtheria bacilli. The feeble resistance of this spirillum to heat and drying makes any interpretation of these granules as spores improbable.

Owing to the recent extensive additions to our knowledge of spiral organisms as agents of disease and the introduction of new methods of culture, the nomenclature is not in a satisfactory state. Most writers on classification have accepted the generic designation vibrio for short, so called comma forms, and spirillum for distinctly spiral forms. The former have one polar flagellum, rarely more, the latter a tuft of polar flagella. The spiral form here described appears, as a rule, in both long spiral and short comma forms in the fetal fluids and may therefore be regarded as standing intermediate between the comma forms and true spirals. Nevertheless since this organism assumes the comma form in its early, most active stage of multiplication in cultures and has single polar flagella, the generic term vibrio appears most appropriate at the present time. It is therefore designated *Vibrio fetus*, *n. sp.* The term spirillum we shall continue to use in the text as a general expression for spiral organisms.

Cultural Characters.

In discussing the culture characteristics of *Vibrio fetus* we must distinguish between the earliest and later cultures. The earliest represent the still unchanged animal type, whereas later cultures may become saprophytized. The change from one to the other growth type goes on gradually and no boundary lines in terms of generations or transfers can be definitely assigned.

The earliest growths have been obtained in agar slants containing a small quantity of condensation water or added bouillon. To this a bit of tissue about $\frac{1}{8}$ gm. or an equivalent amount of stomach or other fluid or intestinal contents of the fetus is added and the tubes are sealed with sealing-wax. In such a medium the growth may be

feeble and is likely to be overlooked entirely at first. After 3 or more days of incubation two very narrow grayish white, opaque lines of growth extend upward from beneath the surface of the condensation water where the agar slant abuts against the glass. These lines, less than 1 mm. broad, are at first the only macroscopic indications of growth. Soon an exceedingly thin veil-like layer extends from each of these two lines into the capillary space between agar and glass, often meeting behind and then forming a complete barely visible layer with upper margins variably high and undulating or jagged, but rarely more than 4 cm. above the bottom of the tube. The condensation water is very feebly clouded but motile spirilla are usually found in it. Surface growth occurs only very exceptionally.

The above features persist in subsequent cultures but there are gradually added others. If defibrinated horse or other mammalian blood is added to the condensation water, a layer of growth appears on the surface of the sedimented corpuscles, which grows heavier with the number of transfers. Surface growths appear after months of cultivation either as grayish white films or as isolated, roundish, smooth, glistening colonies, 1 to 2 mm. in diameter.

With the increasing vigor of growth it becomes possible to obtain multiplication on agar slants without the blood and thereafter the strain may be maintained on plain agar in sealed tubes. In the condensation water a viscid whitish sediment made up of spirilla appears. This may be drawn out into threads. This phenomenon seems to point to a kind of mucoid degeneration which is suggested by the unstained sheaths around spirilla in Fig. 8.

In the earliest growths fluid media failed, as a rule, to induce multiplication. This failure may be due to the continually changing relation to oxygen of the different layers of fluids on account of convection currents. The agar medium provides stable conditions different at different points of the slant. The method of cultivation described differs from the methods of other workers in which deep layers of culture media, either solid, semisolid, or fluid, have been used for bacteria sensitive to oxygen. The use of slanted agar in a sealed tube gives better access to the growth than do the deep layers. It provides various degrees of oxygen tension and hence a greater variety of conditions, one of which may fit the organism to be cultivated.

With some strains a fairly vigorous multiplication has occurred in the first cultures, such as growth on the agar surface and membrane formation on the condensation water, festoons and mucoid shreds hanging down into this fluid. This is due to a specially favorable medium, which is mucus from the stomachs of the fetuses transferred to tubes when original cultures are prepared.

After thirty or more transfers with intervals up to a week multiplication has been observed in unsealed agar slants. The reduced oxygen tension in such tubes is obtained, when necessary to the organism, by growth in the capillary space between the mass of agar and the sides of the tube.

When agar was used in deep layers in sealed tubes the growth phenomena corresponded with those of *Bacillus abortus* under like conditions.

The following description applies to saprophytized strains. Fluid agar, thoroughly mixed with the spirillum, was allowed to set. Growth was gradual, appearing after incubation of 2 to 3 days as a fine white line around the circumference of the surface of the agar, and slowly growing down between the agar and the glass as a fine film. Condensation water gathered, became viscid, and growth spread over the surface of the agar. A dark line forming in the agar below the surface was observed, which at the end of a week's incubation resolved itself into a zone of individual colonies. The colonies were small, opaque, and of a yellow color. In some instances the zone appeared directly under the surface and in others it grew from 2 to 5 mm. below the surface. In unsealed tubes there was no condensation water and no growth at the surface, but the subsurface zone appeared heavier than in the sealed cultures.

In agar stabs growth developed slowly, appearing on the 2nd day along the lines of puncture. The final growth along the stab varied from 5 mm. to 3 cm. in length. If a lateral puncture was made, a fine white film gradually spread between the agar and the glass. At the points of inoculation a surface growth developed, and any slight condensation water present became viscid. In unsealed cultures no growth appeared on the surface.

Coagulated and slanted blood serum (horse) did not prove superior to the slanted agar plus a few drops of blood. No liquefaction was noted in the usually feeble growth. Growth was obtained when

defibrinated blood was incorporated with melted agar and the latter slanted. Multiplication did not take place in nutrient gelatin, either at incubator or room temperature. It is not known, therefore, whether a liquefying enzyme exists or not.

After strains had become adapted to plain slanted agar, attempts were made to induce multiplication in fluid media. Growth in simple beef peptone bouillon in sealed and unsealed tubes becomes possible, but the addition of a few drops of defibrinated blood makes multiplication more vigorous. The medium becomes faintly clouded and a slight viscid sediment is formed. A filmy stringy deposit may settle upon the sides if the tubes are slightly inclined and a viscid ring may appear at the surface, but no pellicle. Growth is not improved when paraffin oil covers the fluid column. In milk no recognizable multiplication occurs.

Biological Characters.

Since *Vibrio fetus* has many biological characters in common with *Bacillus abortus*, it would seem that these two widely separated morphological species are drawn nearer together in their physiological characters through adaptation to the same environment on the fetal membranes, causing the same sensitiveness to oxygen tension, the same fastidiousness towards culture media, and indifference to carbohydrates. Marked differences, however, do occur, such as failure on the part of *Vibrio fetus* to multiply on potato, on which *Bacillus abortus* produces a fairly rich pigmented growth.

The length of time that a sealed original culture at room temperature may contain living organisms differs from tube to tube. One culture gave rise to successful subcultures after 139 days, another failed to do so after 31 days. Subcultures on agar plus blood displayed the same differences—one dead after $2\frac{1}{2}$ weeks, another alive after 9 weeks. It is therefore important in maintaining strains to transfer once a week.

When cultures are stored in refrigerators at a temperature of 5–6°C. above freezing their vitality is reduced considerably as compared with that of cultures kept at room temperature.

The reaction of the bouillon may be carried slightly beyond the neutral point of phenolphthalein without interfering with multiplication. Indole is not formed in bouillon. Fermented bouillon with

1 per cent of dextrose, lactose, and saccharose was used in fermentation tubes to determine gas and acid production. The bulbs became uniformly clouded and a slight deposit developed; the branches remained clear. No gas was produced. After 5 days incubation the bulbs were titrated, and the reaction was found to be neutral or slightly acid. The original reaction of the bouillon had been 0.5 to

TABLE I.

Resistance to Drying.

Strain No.	Temperature.	Time of exposure.	Growth appears in.	Motility in hanging drop.
	°F.		days	
192 (61st transfer)	70–75	1 hr.	3	+
192 (61st ")	70–75	2 hrs.	3	+
192 (61st ")	70–75	3 "		−
192 (61st ")	70–75	4 "		−
192 (61st ")	70–75	5 "		−
192 (61st ")	70–75	6 "		−
192 (61st ")	70–75	1 day.		−
149 (79th ")	70–75	1 hr.	4	+
149 (79th ")	70–75	2 hrs.		−

TABLE II.

Resistance to Heat.

Strain No.	Temperature.	Time of exposure.	Growth appears in.	Motility in hanging drop.
	°C.	min.	days	
192 (70th transfer)	56	5		−
192 (70th ")	56	10		−
192 (71st ")	55	5	3	+
192 (71st ")	55	10		−
149	55	5	3	+
149	55	10		−

0.6 per cent acid to phenolphthalein. The branch showed no acid production.

Vibrio fetus as it occurs in cultures has but little resistance to drying. Bits of linen thread impregnated with fresh active culture material resisted drying 2 hours at room temperature, but not 3 hours, as shown in Table I. Similarly resistance to heat is feeble. It is able to withstand a temperature of 55°C. for 5 minutes (Table II).

Agglutination Reactions.

The scarcity of positive differential characters of the spirilla isolated from fetuses leaves open the possibility that they may not all be alike and that many, if not all, are harmless invaders of the uterochorionic space and the fetal digestive and respiratory tracts. To test the first possibility agglutination with immune serum was resorted to at an early stage of the work. Rabbits were treated repeatedly with doses of living spirilla injected into the abdominal cavity. After 3 to 4 weeks blood was withdrawn and the serum used in the tests shown in Tables III to VI. Before applying the agglutination test it was necessary to await the time when the various strains could be made to multiply on agar slants without blood or bits of tissue so as to eliminate any possible interference on the part of animal tissues.

At this time one of us fortunately isolated a spirillum from the spleen of a young calf which, though culturally like the fetal strains, was nevertheless wholly different in its agglutination affinities. This culture which will be taken up in another publication was used in the tests as a control. It is designated as No. 174. Somewhat later another strain was isolated from the liver of a calf 8 weeks old and the spleen of a guinea pig inoculated with duodenal contents of the same calf. This agrees serologically with *Vibrio fetus* and appears in the tables as No. 321.

The serum used is from rabbits immunized against Strains 67, 174, 256, and 317. The antigens are saline suspensions of the several strains grown on sealed agar slants at 37°C. for 4 days. The salt solution used is 0.85 per cent. The volume of fluid in each final dilution is 2 cc. The culture control contains 1 cc. of the antigen suspension and 1 cc. of salt solution, the serum control 1 cc. of a 1:10 dilution of serum and 1 cc. of normal salt solution. After a period of from 2 to 3 hours at 37°C. readings are taken. The tubes are then placed in the refrigerator over night when the final readings are made.

Tables III to V show that the agglutination reactions are of the usual kind, except for very young strains which yield a more or less paradoxical reaction, in which the clumping is feeble or absent in

TABLE III.

A. Serum from a Rabbit Treated with Cultures of Vibrio 67.

Vibrio No.	No. of transfer.	Dilutions of serum.											Controls.	
		1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240	1:20,480	Culture.	Normal serum.
67	64	C.*	C.	C.	C.	C.	C.	C.	+++[1]	+++[1]	++[1]	++	+	–	–
149	75	"	"	"	"	"	"	"	C.	+++[1]	+++	++[1]	++	–	–
159	72	"	"	"	"	"	"	"	"	C.	+++[1]	+++	++	Settling and clumps; cloudy.	–
174		–	–	–	–	–	–	–	–					–	–
179	71	C.	C.	C.	C.	C.	C.	C.	C.	C.	+++[1]	++[1]	++	–	–
192	?	"	"	"	"	"	"	+++[1]	+++	+++	++[1]	++	++	–	–
213	56			"	"	"	"	C.	C.	++[1]	++[1]			–	–
246	25			"	"	"	"	+++[1]	+++	++	+			–	–
251	23			+++[1]	"	"	"	+++[1]	+++	+++	++			–	–
256	21			C.	"	"	"	+++[1]	+++[1]	+++	+++			–	–
258	19			"	"	"	"	++	+	+	+			–	–
263	14			"	"	"	"	C.	C.	C.	+++			–	–
267	17			–	–	–	+++	+++[1]	"	++[1]	++			–	–
289	8		C.	+++	+++	+++	+++	++	++	++	–	–		–	–
290	8		"	C.	C.	C.	++	++	+	+	+	–		–	–

* In this and the following tables C. indicates complete agglutination, +++ nearly complete agglutination, ++ marked agglutination, + slight agglutination, ± doubtful, and – no agglutination.

[1] when following a symbol denotes a degree of agglutination between it and the next higher symbol.

TABLE III—*Concluded.*

B. Serum from a Rabbit Treated with Cultures of Vibrio 67.

Vibrio No.	No. of transfer.	Dilutions of serum.								Controls.	
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	Culture.	Normal serum.
67	90	C.	C.	C.	C.	C.	C.	+++[1]	−	−	−
149	97	"	"	"	"	"	"	C.	C.	−	−
318	17	"	"	+++[1]	+++	+++	++	+	+	−	−
159	96	"	"	C.	C.	C.	C.	C.	C.	−	−
179	89	"	"	"	"	"	+++	++	+	−	−
289	25	"	"	"	"	+++[1]	+++	++[1]	++	−	−
317	18	"	"	"	"	C.	C.	C.	+++[1]	−	−
308	21	"	"	"	"	"	"	+++[1]	++	−	−
317	19	"	"	"	"	+++	++	+	±	−	−
267	39	"	"	"	"	C.	C.	+++[1]	+++	−	−
321	17	"	"	+++[1]	+++	++	+	−	−	−	−
321	13	"	"	C.	C.	+++[1]	+++	++	+	−	−
331	4	+++	+++	+++	++	+	−	−	−	−	−
333	3	++	++	++	++	++	++	−	−	−	−
339	3	+	++	++	++	++	+	+	+	−	−

TABLE IV.

Serum of a Rabbit Treated with Cultures of Vibrio 256.

Vibrio No.	Dilutions of serum.								Controls.	
	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	Culture.	Normal serum.
67	C.	C.	C.	C.	C.	+++[1]	+++	+++	–	–
149	"	"	"	"	"	C.	+++[1]	+++[1]	Slight deposit; cloudy	–
159	"	"	"	"	"	"	+++[1]	+++[1]	" "	–
174	–	–	–	–	–	–	–	–	–	–
179	C.	C.	C.	C.	C.	+++[1]	+++	+++	–	–
192	"	"	"	"	"	C.	+++[1]	+++[1]	–	–
213	"	"	"	"	"	"	+++[1]	+++[1]	–	–
246	+++	"	"	"	"	"	C.	+++	–	–
251	–	–	+++	+++[1]	+++[1]	"	+++	+++	–	–
256	C.	C.	C.	C.	C.	"	C.	+++	–	–
258	"	"	"	"	"	"	+++[1]	+++[1]	–	–
263	–	+++	"	"	+++	++	+[1]	+	–	–
267	–	–	+	++	+++	C.	+++	++	–	–
289	+	++	+++	C.	C.	+++	++	–	–	–
290	–	–	+	+++	"	C.	+++	++	–	–
321a*	+++[1]	+++[1]	++	+	–	–	–	–	–	–
321b†	C.	C.	C	+++	+++	+	–	–	–	–

* Liver strain.

† Guinea pig passage strain.

TABLE V.

Serum of a Rabbit Treated with Cultures of Vibrio 317.

Vibrio No.	No. of transfer.	Dilutions of serum.								Controls.	
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	Culture.	Normal serum.
317	20	C.	C.	C	C.	C.	++[1]	+++[1]	+++	Slight sediment.	–
67	92	+++	+++[1]	+++[1]	++	±	–	–	–	–	–
174	95	–	–	–	–	–	–	–	–	–	–
213	71	C.	C.	C.	C.	C.	+++	++	+	–	–
267	42	"	"	"	+++	++	+	±	±	–	–
318	21	"	"	"	C.	+++	++	+	–	–	–
290	25	"	"	"	+++	++	+	±	–	–	–

TABLE VI.

Serum of a Rabbit Treated with Cultures of Vibrio 174.

Vibrio No.	Dilutions of serum.												Controls.	
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240	1:20 480	Culture.	Normal serum.
67	—	—	—	—	—	—	—	—					—	—
149	—	—	—	—	—	—	—	—					—	—
159		—	Slight clearing.	Slight clearing.	—	—	—	—					Slight deposit; cloudy.	—
174	C.	C.	C.	C.	C.	C.	C.	C.	C.	C.	C.	+++[1]	—	—
179		—	—	—	—	—	—	—	—				—	—
192		—	—	—	—	—	—	—	—				Slight deposit.	—
213		—	—	—	—	—	—	—	—				—	—
246		—	—	—	—	—	—	—	—				—	—
251		—	—	—	—	—	—	—	—				—	—
256		—	—	—	—	—	—	—	—				—	—
258		—	—	—	—	—	—	—	—				—	—
263		—	—	—	—	—	—	—	—				—	—
267		—	—	—	—	—	—	—	—				—	—
289		—	—	—	—	—	—	—					—	—
290		—	—	—	—	—	—	—					—	—
308*		—	—	—	—	—	—	—	—				—	—
321a		—	—	—	—	—	—	—	—				—	—
321b		—	—	—	—	—	—	—	—				—	—
174		C.	C.	C.	C.	C.	C.	C.	C.				—	—

* This and the following tests were made at a later date.

the highest concentration and rises to a maximum to fall again. The older strains present the usual features of complete sedimentation with clarification of the supernatant fluid in the high concentrations, and gradual increase of clouding of the fluid in the lower ones. The tables indicate a close relationship between Strains 67, 256, and 317, if not identity, as the word is used in establishing a definite species of bacteria. Furthermore, the calf strain, No. 174, shows no agglutination affinities whatever with the fetal strains or with the other calf strain, No. 321.

SUMMARY.

Twenty-two fetal and two calf strains of spirilla have been studied chiefly with regard to the problem of identity. Twenty-one fetal strains are probably specifically the same. One fetal strain differs slightly from these, but in its agglutination affinities it belongs to the same group. Of two strains isolated from calves one has definite agglutination relations with the fetal strains, the other none. In the morphological and biological characters so far investigated all the strains agree closely with one another.

EXPLANATION OF PLATE 16.

Magnification, × 1,000.

FIGS. 1 to 3. *Vibrio fetus* (No. 159) stained to show the polar flagella.

FIG. 4. *Vibrio fetus* (No. 213) stained in the same way.

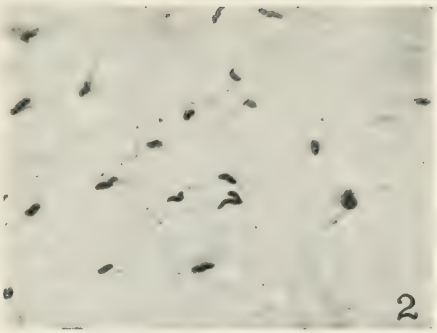
FIG. 5. Spirillum from a calf (No. 174) stained in the same way.

FIG. 6. Preparation from a culture of *Vibrio fetus* (No. 290), 10th transfer, showing long and short forms, some stained deeply and with granules, others more faintly. Dilute aniline water gentian violet.

FIG. 7. Preparation from a culture of *Vibrio fetus* (No. 333) showing long forms. Stain as in Fig. 6.

FIG. 8. Another field of the same preparation showing some capsular substance, unstained, sheathing the long forms.

1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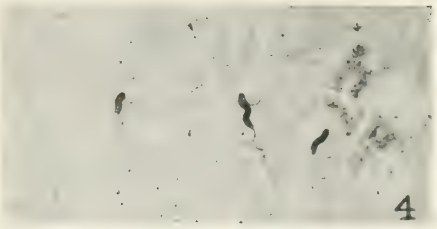
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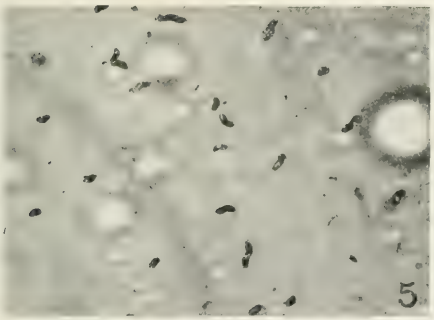
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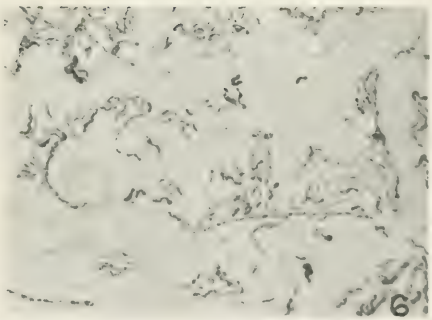
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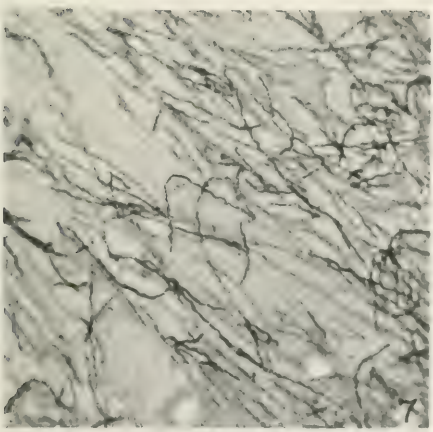
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THE ETIOLOGICAL RELATION OF SPIRILLA (VIBRIO FETUS) TO BOVINE ABORTION.*

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Since the summing up of the data contained in a former communication on this subject[1] investigations have been continued upon material from the same herd. From August, 1918, to June, 1919, twelve additional cases of abortion in which spirilla were isolated from fetuses in pure cultures have been studied, making twenty-six in all. In one case a slightly divergent strain was present, whose significance cannot be defined. It appears, as a rule, in long filaments, although short forms are not lacking. Thus far, motility has not been detected. The case from which this form was isolated is included in Table I as No. 308. In another paper[2] data on the agglutination affinities of the various strains are given and it is there shown that this aberrant strain is serologically related to the regular type. The reader is also referred to an earlier paper[1] on culture methods employed. Emphasis needs to be put on the importance of cultures from the digestive tract and the lungs. If the fetus after expulsion breathes and swallows, both tracts are likely to be contaminated with miscellaneous bacteria and spirilla will be suppressed in the cultures. Even when the fetus is dead when expelled, if the mouth and nose should lie in water or other fluids these tracts may become contaminated. Cultures from spleen, liver, and kidneys of the fetus by themselves

* This and the three accompanying papers are interrelated inasmuch as the fundamental data are the same. Duplication of statements has been avoided as far as possible and the papers should therefore be considered together in estimating the accuracy of the conclusions formulated.

[1] Smith, T., *J. Exp. Med.*, 1918, xxviii, 701.

[2] Smith, T., and Taylor, M. S., *J. Exp. Med.*, 1919, xxx, 299.

TABLE I.

Fetuses from Which Spirilla Were Isolated. Guinea Pig Tests for the Presence of B. abortus.

Cow No.	No. of sire.	Length of fetus.	Date of abortion.	No. of pregnancy after purchase.	Cultures.							Guinea pig inoculations.			
					4th stomach.	Rectum.	Lung.	Spleen.	Liver.	Kidney.	Fetal fluids or swabs.	4th stomach.	Lung.	Meconium.	Placenta or uterine discharge.
		in.	*1918*												
289	8, 6, 2	20	Nov. 18	2	++* (Rumen ++.)	++	+	−−	−−	++		−	−	−	−
290	4, 9	24	" 19	3 (native).	+	+	++	++	++	++		−	−	−	−
308	?	31½	Dec. 30	1	−−	+++ (Small intestine −.)	++	−−	−−	−−		−	−	−	−−
			1919												
317	9	25	Jan. 27	2	+ and other forms.	++	++					−	−	−	
318	3, 7	26	" 27	2	++	+ and other forms.	+ and other forms.					−	−	−	−
331	9	8	Mar. 15	6 (native).	Two pure cultures of spirilla; one culture of paratyphoid; one culture of cocci and spirilla.							−			−−
333	2, 10	26	" 27	1	++	++ (Colon ++.)	++ and streptococci.	−−	− and bacilli.	−−		−	−	−	
339	1, 2	35½	Apr. 9	3	−− (Films +.)	−− (Films of small intestine +.)	++	−−	−− (*B. pyogenes* +.)	−−		−	−	−	−

347	2	25½	Apr. 23	4	++ (Rumen ++.)	++	++	+	−−	−−		−	−		−
351	7, 11	28	May 5	1	++ and *B. abortus.*	++	Miscellaneous bacteria.	+ and *B. abortus.*	Miscellaneous bacteria.	+ and *B. abortus.*		−	+	−	+
356	4, 8	26½	" 23	1	+++ (One tube contains a bacillus also.)	++ (Colon +.)	++		++	++		−	−	−	−
357	9	21¾	" 24	8 (native).	++ (Small intestine +.)	++ (Colon +.)	+ and contaminated.	Contaminated.	−	−	(Film +.)	−	−	−	−

* Each + stands for a pure culture of spirilla unless otherwise stated, each − for a negative culture or negative guinea pig test with reference to *B. abortus.*

cannot be depended on to bring out the presence of spirilla in the fetus.

The notes on the pathological changes in the fetuses are omitted, since they do not differ from those of the earlier cases reported.[1] No single abnormal condition or group of conditions in the fetus could be used to prognosticate the nature of the infecting organism.

In most cases the placenta was retained. In one (No. 289) it was reported to have been discharged but it was not preserved. A portion of the placenta of No. 318 was passed attached to the fetus. It represents about one-third of the entire mass. About one-half is markedly diseased. The cotyledons are of a dirty whitish color and covered with a thin deposit which is readily washed off by repeated baths of normal salt solution. The villi after washing stand up like normal villi but lack entirely the blood-red color. Towards the more normal cotyledons some are found which contain abnormal, whitish villi and tufts or clumps of the same among the normal blood-red ones.

The intercotyledonous chorion, where cotyledons are diseased, is usually thickened and roughened. The surface is mapped out into small 1 to 2 mm. slightly raised plaques. These feel like thickened epidermis. When scraped only very little comes away. The underlying tissue is more or less edematous in localized areas, usually around and under a diseased cotyledon. Films of the cheesy covering of cotyledons stained 10 minutes and 24 hours show numerous spiral forms with from $\frac{1}{2}$ to 4 or 5 complete turns. They are all of the same fineness as regards diameter, but the amplitude of curves and the form vary somewhat. This may be due to the drying.

Sections of fixed and hardened tissue show besides a general quite marked edema of the chorion, localized masses of nuclear debris under the epithelium, now lost. This nuclear debris results from dense collections of leucocytes, probably polynuclear, which evidently form the hard plaques of the intercotyledonous areas of the chorion, mentioned above. There is, furthermore, a general diffuse infiltration of cells into the chorion. The cotyledons contain necrotic villi without cell infiltrations.

It was stated in the preceding article[1] that *Bacillus abortus* could not be detected in the fourteen cases from which *Vibrio fetus* was iso-

lated. The same is true of those tabulated here, with the exception of one case, No. 351. In cultures from this there were found for the first time both spirilla (vibrios) and *Bacillus abortus*. The former were present in cultures from contents of the fourth stomach, colon, rectum, spleen, and kidneys. *Bacillus abortus* appeared several days later in the fourth stomach, spleen, kidney, and rectum cultures. The tubes with contents of the colon contained only spiral organisms.

The absence of *Bacillus abortus* from such a large percentage of cases is of importance. The dropping of immature calves and fetuses of various ages can no longer be referred to *Bacillus abortus* without a bacteriological study of the fetus and placenta if obtainable, or else of uterine discharges obtained with swabs and examined microscopically and by inoculation of guinea pigs. Outbreaks of abortion due solely to *Bacillus abortus* very probably occur when a herd is first attacked and therefore highly susceptible. After a time the acquired immunity modifies the conditions materially and *Bacillus abortus* becomes a relatively unimportant factor in all but first pregnancies.

Vibrio fetus may be either a true agent causing disease of the fetal membranes, or it may be simply an invader from the more external genital tract, or from the blood after the fetus has been injured or killed by other non-bacterial agencies. The simplest method to prove the truth of one or the other hypothesis is to inoculate pregnant cows. This procedure is not so easily carried out, however. In addition to the difficulties encountered in obtaining non-immune and non-infected pregnant cows is the probable attenuation of the vibrio under cultivation. This, as is described elsewhere, is subject to changes under cultivation, such as a tendency to form mucoid masses, and an adaptation to culture media without blood or bits of tissue. The quantity of growth tends to increase with successive transfers. Changes in growth characters as extensive as these most probably involve other changes, among which virulence stands first. Unfortunately, laboratory animals have so far proved refractory and cannot serve as tests of virulence. If very early transfers are inoculated into cows, the danger of introducing some hypothetical ultramicroscopic virus is much greater than in later transfers. Filtration

does not remove this possibility, since the minute spiral organisms, especially the short forms, might pass through the pores of the filter. With these difficulties in view the writer has been able to test *Vibrio fetus* upon four pregnant cows. The records of these cases are as follows:

Cow 295.—Received Nov. 21, 1918. Black Holstein of unknown age, supposed to be pregnant, according to physical examination. Reacts to tuberculin.

Dec. 10. Cow received into the left jugular vein the following suspension. Four agar slants, sealed, of vibrio (Cow 267, 17th transfer, inoculated Dec. 6) were used. The condensation water was first removed with pipette, then the agar moved about after adding some fresh bouillon to dislodge organisms growing between agar and glass. In all, about 10 cc. of a faintly clouded fluid were obtained. A hanging drop of this fluid shows rather long spirilla with feeble motility. Before the date of the injection the maximum range of temperature during the day was from 38.4°C. at 8 a.m. to 39.4°C. at 5.15 p.m. On the day of injection, which was made at noon, the late afternoon temperature was 40.6°C. On the following day it had fallen to normal.

On the assumption that the culture injected might have lost some of its virulence a second injection of a more recently isolated strain was made Jan. 8, 1919. Growth from sealed agar slants (Fetus 289, 7th transfer, 6 days old) was suspended in plain bouillon. About 9 cc. of a feebly clouded fluid were injected into a jugular vein. There was hurried respiration following the injection and some coughing. The maximum afternoon temperature was 39.8°C. Between the date of injection and the birth of a calf on Feb. 6, the morning temperature was either slightly higher than the evening temperature or else the same. The maximum morning elevation was 39.5°C., the usual temperature 39–39.3°C.

Feb. 6. After normal labor cow gave birth to a living calf. The placenta was still retained after 24 hours and the major portion of it was removed manually.[3] A thick, turbid, reddish discharge with small coherent mucoid masses in it was caught in a sterile bottle by the attendant several hours after birth of calf. Fresh and stained films showed presence of cells resembling polynuclear leucocytes but with chromatin chiefly on margin and central portion nearly unstained and in most cases containing a small number of organisms, some of which were distinctly spiral, others of same diameter, but spiral form not demonstrable in them. The groups of leucocytes were in part held together by amorphous masses staining blue. Many isolated spirillar forms of one or two wave lengths present. Other bacteria scarce.

Of the placenta removed Feb. 7, the intercotyledonous areas of chorion have the minute network of vessels conspicuously injected. Small masses of a yellowish exudate, of pin-head size, sprinkled over the surface. These masses con-

[3] The placenta was removed by Dr. Ernest W. Smillie and Dr. Ralph B. Little.

sist of amorphous material staining blue, remnants of cells and miscellaneous bacteria. The subchorionic tissue is not edematous. Besides these abnormal conditions, the chorion presented several areas which were thickened, opaque, whitish and slightly nodular, firm and leathery. The cotyledons vary much in appearance. None has the blood-red color of the normal mature placenta. In all there are yellowish areas, occupying more or less of the cotyledon and supplanting the normal color. This yellowish color is due largely to deposits or exudates like those described above. When a cotyledon is rinsed repeatedly in normal salt solution, the wash water becomes heavily clouded and filled with flocculi. After three or four changes these are removed and there remain in the cotyledon a variable number of yellowish villi among the dark or light red ones, which are the result of necrosis. These may form an outer zone only or occupy most of the cotyledon. In none of the many films examined were bacteria resembling *B. abortus* found. Up to the time this cow was removed for slaughter, Feb. 12, a slender mass of placental tissue about 2 feet long remained hanging from the vulva. Unfortunately, through some mistake at the abattoir, the uterus was not reserved.

In sections from the placenta there is a complete loss of chorionic epithelium. There is extensive cell infiltration with subsequent necrosis and nuclear disintegration in foci along the subepithelial layer of the chorion and in the villi. These and their branches are densely filled with nuclear debris. Occasionally a vessel is recognizable, also densely filled. The appearances indicate an active polymorphonuclear infiltration followed by necrosis. Microorganisms not definitely seen.

The calf of No. 295 appeared somewhat under weight at birth. On the 4th day it weighed 75 pounds. At birth the feet were somewhat soft and hoofs not fully developed. In all other respects the calf was normal and remained free from scours during the 6 days it was under observation.

To determine whether this cow might harbor *B. abortus* as a cause of the disease of the placenta, two guinea pigs were inoculated with uterine exudate obtained several hours after birth of calf. Both guinea pigs were normal when killed 48 days after inoculation and cultures were negative Before the first inoculation on Nov. 28, samples of blood were withdrawn from jugular vein and the serum was tested for specific agglutinins towards *B. abortus*. Only in dilution of 1: 10 was clumping observed. On Jan. 27, a second sample was withdrawn. This also was negative. Tested against *Vibrio fetus*, a very good agglutination was obtained with two older strains but not with recent strains, a condition quite regularly observed (Table II).

On Dec. 2, 1918, about 100 cc. of milk were obtained from the udder. To this were added 50 cc. of salt solution and the mixture was centrifuged at high speed for 20 minutes. Three guinea pigs were inoculated by injecting 7 cc. of the deposit and lowest strata of fluid into the peritoneal cavity of each. After 57 days the animals were killed with chloroform. There were no lesions suggestive of the presence of *B. abortus* and the cultures from the spleens remained free from growth.

The evidence is thus quite conclusive that *Bacillus abortus* was not responsible for the lesions. The microscopic examination of the early postpartum discharges showed many spirilla both free and within leucocytes. The placenta was frankly diseased and the character of the lesion, infiltration and exudation of leucocytes, corresponded with the lesions found in earlier cases, associated with spirilla. The following case is not so convincing as to the rôle of spirilla in producing disease of the chorion but the evidence is against an infection with *Bacillus abortus*.

TABLE II.

Cow 295. Agglutination Test.

Strain from Cow No.	No. of generation or transfer.	Dilutions of serum.*							
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560
67	78	C.†	C.	C.	C.	C.	C.	+++	++
179	81	+++	"	"	"	"	+	−	−
267	25	−	−	+	−	−	−	−	−
289	11	−	−	−	−	−	−	−	−

* Serum from blood withdrawn Jan. 27, 1919. The tubes were kept at 37°C. for 3 hours, then in the refrigerator at about 6°C. over night.

† In this and the following table C. indicates complete clumping, and clearing of the fluid, − no change. The plus signs represent grades of clumping.

Cow 296.—White Holstein, obtained with No. 295. Assumed to be pregnant according to physical examination. Reacts to tuberculin.

Beginning Nov. 25, 1918, the temperature was taken twice daily. The highest morning temperature up to Dec. 10, the day when the animal was inoculated, was 39.2°C., the highest evening temperature 39.6°C. On Dec. 10, the growth in four sealed agar slants of spirilla from Cow 289, 4th transfer, 4 days old, was suspended in bouillon. About 10 cc. of a faintly clouded suspension were injected into the left jugular vein at noon. On this day the late afternoon temperature was 41.2°C. Next day the temperature was normal. Attendant reported violent jerky movements in abdomen of cow.

A normal calf was born early Dec. 19, 9 days after inoculation. At 9 a.m. the placenta was not completely discharged. It extended from vulva to floor, (the animal standing) where most of it lay in the bedding. A portion, including half a dozen cotyledons, which had not touched the floor was removed for examination. The intercotyledonous areas of chorion have all vessels conspicuously injected. The larger ones are cyanotic in appearance. Resting on these areas are soft, orange-colored masses of pin-head size which crush like soft cheese

between cover-slips. They consist of cell debris, fat globules, and probably leucocytes. When stained, these masses are seen to consist of polynuclear leucocytes, some epithelium, and cell debris, bound together with a cement-like substance, staining blue in alkaline methylene blue. A few spirilla-like forms detected in films of these masses after considerable search. The cotyledons in general are free from necrotic villi and the latter appear normal when covered with water. Over a number of cotyledons, however, there is the same flaky exudation, even more abundant than over the intercotyledonous areas. Films of this exudate show a few curved forms besides the cellular and amorphous masses already described. Sections of fixed and hardened portions of placenta indicate in general a still normal tissue. In slides from four different regions, however, there are a few small foci of dense polynuclear cell infiltration. One of these surrounds a thrombosed vessel in a villus.

Cultures from the exposed placenta became overgrown with a variety of bac teria. Spirilla not detected in the mixed growth. On Dec. 25, pasty, whitish, semitranslucent masses were found in bedding. These probably came from the uterus. Films showed pus cells and immense numbers of minute cocci and rod-like forms.

In order to determine whether *B. abortus* was associated with the placental lesions, three guinea pigs were inoculated with scrapings of the placenta, ground in sand and suspended in salt solution. After 43 days they were killed. There were no lesions suggesting *B. abortus* and the spleen cultures remained clear.

To determine still further the presence or absence of *B. abortus* a sample of blood was collected Nov. 28, and the serum tested against *B. abortus*. Only in a dilution of 1: 10 was clumping recognizable. A second sample withdrawn Jan. 13, 1919, gave a similar result. The titer of the same serum was tested against four strains of spirilla (Table III).

On Dec. 2, 1918, about 50 cc. of milk were obtained from the udder, centrifuged, and of the lowest layer about 15 cc. were injected into the peritoneal cavity of three guinea pigs, 5 cc. into each animal. One guinea pig died in 39 days. There was extensive plastic peritonitis causing adhesion to one another of all the abdominal organs. The two remaining animals were killed with chloroform in 57 days and were found normal. Cultures from spleen remained free from growth.

The two following cases, both inoculated with strains of spirilla, were entirely negative.

Cow 334.—Received Mar. 20, 1919. Jersey grade, about 7 years old. Pregnant according to physical examination. Reacts to tuberculin.

Mar. 31, noon. Cow received into left jugular vein a suspension of spirilla isolated from Fetus 331. The cultures used were of the 3rd generation (or transfer) in sealed tubes of slanted agar containing bits of guinea pig spleen and some calf serum water. Growth after 4 days incubation feeble, but charac-

teristic. It was washed off with salt solution, bouillon added to 4 cc., and gently centrifuged to throw down minute clumps of agar, etc. The resulting suspension contained active spirilla. The temperature taken twice daily from the time of arrival fluctuated between 38° and 39°C. except on the afternoon of the day of injection, when the late afternoon temperature was 40.6°C.

An agglutination test against *B. abortus* was feebly positive at 1:20. The cow was kept under observation until Apr. 28, when she was slaughtered. The fetal membranes were normal in appearance. The fetus was 31 inches long. The only departure from the normal was the presence of a small quantity of reddish, clear fluid in the thoracic and abdominal cavities and a slight interlobular edema of the lungs. Cultures from fetal fluids (amniotic, allantoic, scrapings from chorion) and from fourth stomach of fetus were sterile after 2 weeks of incubation.

TABLE III.

Cow 296. Agglutination Test.

Strain from Cow No.	No. of generation or transfer.	Dilutions of serum.*								Controls.	
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	Culture.	Serum.
67	79	C.	C.	C.	C.	+++	−	−	−	−	−
179	81	"	"	"	"	+++	−	−	−	−	−
267	24	−	−	−	−		−	−	−	−	−
289	10	+	+	+	−	−	−	−	−	−	−

* Serum from blood drawn Jan. 13, 1919. Tubes kept at 37°C. for 3 hours, then in refrigerator at about 6°C. over night. The control culture was suspended in salt solution. The serum control contained 5 per cent serum.

Cow 335.—Received with No. 334. Jersey grade, 9 to 10 years old. Pregnant. Reacts to tuberculin.

Mar. 31, noon. Inoculated with spirilla from Fetus 317, 13th transfer, on slanted agar. The cultures were 4 days old. Growth fair. The spirilla were removed in the same manner as for Cow 334. Active spirilla present in the final suspension. The injection was made into the left jugular vein. Within a few minutes the cow began to breathe rapidly and cough. This lasted about 2 hours. The temperature, which had been fluctuating between 38.4°C. in the morning and 39.4°C. in the late afternoon, rose to 40.6°C. on the day of injection and was normal again next day. On the same day a slight discharge from vagina was noted. A guinea pig inoculated with it showed no disease, 6 weeks later.

Agglutination tests made with *B. abortus* on Mar. 27 showed a positive clumping up to dilutions of 1:160. The cow was slaughtered with No. 334, Apr. 28, and the uterus with contents removed to the laboratory. There was no evidence of disease of the chorion. Cultures from the several fluids (allantoic, amniotic) and scrapings from chorion were negative. The fetus was 26¾ inches long. Its

organs were normal with the exception of marked edema of the walls of gall bladder and the mesentery of duodenum carrying the common bile duct. Cultures of fourth stomach fluids remain sterile.

A sample of milk obtained Mar. 27 and tested on guinea pigs was later shown to be infected with *B. abortus*. This explains the agglutination titer of 1:160 towards *B. abortus* and makes it certain that this cow had passed through the abortion disease in some earlier pregnancy. It also shows that the udder may be infected with *B. abortus* and the fetal membranes and fetus remain normal. The agglutination reactions of the blood of Cows 334 and 335, taken Apr. 24, towards their own and one other strain of spirilla were quite irregular and the results are therefore withheld for further study of this test.

The foregoing experimental tests of the pathogenic rôle of *Vibrio fetus* cannot be regarded as final, but as far as they have been carried they give strong evidence that it is an etiological factor in bovine abortion. Spiral forms from four different cases were inoculated (Nos. 267, 289, 317, and 331). Only the two cases inoculated with those from Case 289 may be considered positive. Whether this negative outcome in the two remaining cases is due to decline or absence of virulence or to varying resistance on the part of the cows must be determined by additional experiments of the same kind.

SUMMARY.

The isolation in pure culture of a definite morphological entity (vibrio or spirillum) with practically the same biological characters from a series of cases of the same clinical complex (abortion) establishes a presumption in favor of the specific identity of the organisms and also in favor of the inference that such organisms are etiologically related to the diseased condition. This presumption is strengthened by the fact that disease of the fetal membranes followed the injection of pure cultures of this organism in two out of four cases.

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THE BACTERIOLOGY OF BOVINE ABORTION, WITH SPECIAL REFERENCE TO ACQUIRED IMMUNITY.

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Since the general confirmation of Bang's discovery of a characteristic bacillus, *Bacillus abortus*, associated with abortion in cattle in different countries and the successful production of disease of the fetal membranes through infection of pregnant cows with *Bacillus abortus* in pure culture, it has been taken for granted that Bang's bacillus was the sole infectious agent, and further research into the etiology has given way to the study of methods of diagnosis, of vaccines, and their application.

The bacteriological study of cases of abortion as they occurred in a large herd in which a fair proportion of the stock was bred on the spot with accessions from other herds from time to time, continued over a period of $2\frac{1}{2}$ years, has shown that while *Bacillus abortus* may be the sole agency of abortion in certain herds, this is clearly not true for the herd under investigation.

In view of this somewhat unexpected result it seemed desirable to go over the data pertaining to the various cases in some detail, first, to see how far the bacteriological results could be depended upon to give information concerning the nature of the infectious process leading to death and expulsion of the fetus, and second, to note the bearing of the bacteriological data on the acquisition of immunity by the cow against later infection.

Methods.

The methods used for determining the presence or absence of infectious agents in the fetus and fetal membranes were those directed towards finding *Bacillus abortus* and spirilla (*Vibrio fetus*). Bits of

tissue from lungs, spleen, liver, and kidneys, and several drops of fluids from the different sections of the digestive tract of the fetus were added to slanted agar and the tubes sealed with sealing-wax. At the same time guinea pigs were inoculated with salt solution suspensions of contents of the digestive tract, portions of lungs, fetal membranes, and of swabs from the uterus whenever obtainable. For the details of the method of using guinea pigs and the results obtainable, the reader is referred to the communication by Smillie.[1]

In general, cultures will not succeed unless the amounts of fluid or tissue transferred to culture tubes are adequate. Usually bits of tissue the size of small beans or fluids of equivalent amount are sufficient. But even with these the growth may be limited to the condensation water and the adjacent portion of the slant on account of the few bacteria contained in the material.

As to the relative delicacy of the two methods—cultures direct from the fetus and guinea pig inoculations—in revealing the presence of a very few abortion bacilli, it may be stated that in most cases direct cultures and guinea pig inoculations agreed in being either both positive or both negative. In a few cases, however, in which the inoculation of guinea pigs failed to produce either manifest lesions or positive cultures from the spleen, the direct cultures showed the presence of a very few abortion bacilli. The method of direct culture may therefore be considered superior to the guinea pig test under conditions assuring pure cultures.

Under other conditions the use of guinea pigs must replace largely the cultures. Fetuses over 7 months old may be born alive. In that case they inspire a little air and swallow some saliva or bedding before they die. The invasion of miscellaneous bacteria goes on very rapidly and cultures with lung tissue or fluids from the stomachs will usually be overgrown with a variety of forms. Guinea pigs inoculated with such material may show the presence of *Bacillus abortus* unless the invasion has gone too far, when the guinea pigs may rapidly succumb to a miscellaneous infection in which pathogenic anaerobes figure largely.

To obtain pure cultures of *Bacillus abortus* from the fetal mem-

[1] Smillie, E. W., *J. Exp. Med.*, 1918, xxviii, 585.

branes, the indirect method of inoculating guinea pigs is usually the only one successful, unless the placenta is promptly discharged and, if soiled, the diseased portions are repeatedly washed in sterile water or salt solution. This procedure removes much of the infection itself in the shape of infected epithelium and phagocytic cells, but it leaves enough to make isolation of *Bacillus abortus* possible. The material is transferred to an agar slant and well distributed over the surface. A loopful from this is transferred to a second, and so on to a third or fourth and the tubes are sealed.

When the placenta remains within the uterus 24 hours or longer, the miscellaneous infection becomes so great that guinea pigs inoculated with uterine fluid or suspensions of placental tissue rarely survive a septic infection resembling that due to malignant edema or *Bacillus welchii*, unless very small doses are injected and three or four guinea pigs used. Retention of the placenta *in utero* following abortion is the rule, and therefore cultures and guinea pig inoculations of discharges are likely to fail because of the predominance of other bacteria. Even when the placenta is discharged in part it may be in a condition which precludes the successful isolation of *Bacillus abortus*.

The question then arises how far the results obtained from cultures and guinea pig tests of fetal tissues and fluids can be depended on to assure a diagnosis. In other words, is the fetus invariably infected with *Bacillus abortus* when the placenta is involved? If not, what is the percentage of abortions in which *Bacillus abortus* fails to be demonstrable in the fetal organs by the method described?

This, like other questions involving bacteriological methods, can be answered only through cumulative data. It is impossible to subject the entire fetus to bacteriological tests, and the bacteria may be so scarce or so unevenly distributed in the digestive and respiratory tracts as to escape the culture tube. The data on which this paper is based contain a small percentage of cases in which both fetus and placenta were cultured and tested on guinea pigs. This material will be fully discussed in another publication, and we can merely give results here which go to show that in perhaps not more than one out of twenty-five cases were cultures from the fetus negative and guinea pig inoculations from placental tissue or uterine discharges positive.

It may on the whole be accepted, therefore, that only a very small proportion of abortions due to *Bacillus abortus* occur before the fetus has been invaded. Even in the case of certain fetuses born alive which survived from 1 to 7 days, *Bacillus abortus* was isolated from the lungs through guinea pigs. Owing to the presence of septic organisms the intestinal contents in such cases cannot be inoculated into guinea pigs without inducing a rapidly fatal septic infection in most animals.

Grouping of Cases According to the Associated Bacteria.

In the tables the cases, for convenience, have been divided into four groups: (1) the cows raised within the herd, from which *Bacillus abortus* was isolated; (2) the cows introduced into the herd from without, from which *Bacillus abortus* was isolated; (3) cases of infection with *Vibrio fetus*; (4) cases of abortion in which cultures remained sterile and guinea pig inoculations negative, or which were associated with other agencies such as *Bacillus pyogenes*.

In Table I there are 37 cases from which *Bacillus abortus* was isolated. Of these, 26 were first pregnancies, 7 were second pregnancies, 3 were third pregnancies, and 1 was a fourth pregnancy. Of the cows aborting in the second pregnancy, 4 aborted both times and 1 had a normal first gestation. Of the 3 cows aborting in the third pregnancy, one is recorded as having aborted in the second pregnancy. The other 2 have no record of previous abortions. One aborting in the fourth pregnancy has no record of earlier abortions.

In Table II are brought together all purchased cows whose fetuses or calves or fetal membranes were found infected with *Bacillus abortus*. Some of these cows had given birth just before purchase. Some gave birth soon after purchase. In the third column are given all calvings between the time of introduction into the herd and the abortion or premature delivery of calves. If the period between acquisition and the first calving thereafter is a month or less, the next pregnancy may be considered the first in which infection with *Bacillus abortus* became possible in the new surroundings. In such cases, therefore, the second pregnancy has been designated the first in the table.

TABLE I.

Native Cows, Aborting or Giving Birth to Living Calves from Which B. abortus Was Isolated.

Case No.	Length of fetus.	No. of calving.	Remarks.
2	46 cm.	2	Aborted both times.
16	Destroyed.	1	Placenta inoculated.
17	27 in.	1	
19	Calf alive.	2	
21	25 in.	1	
22	23 "	1	
26	?	1	
30	Nearly mature.	3	2 wks. premature. No record of earlier abortions
31	27 in.	1	
33	26 "	1	
41	Nearly mature.	1	
78	28 in.	1	
86	21½ "	1	
89	Alive.	1	
105	19½ in.	3	Aborted also in second pregnancy.
120	14 "	2	First calf normal.
134	Calf alive.	1	Placenta inoculated.
146	Fetus not found.	1	" "
150	30½ in.	1	*In utero.*
153	2 cm.	2	
164	Alive.	1	
180	33 in.	1	Fetus lived several hrs.
200	21 "	1	
203	Alive.	1	
206	28 in.	1	
210	Calf alive.	1	
215	" "	1	
224	" "	1	
227	" "	1	
229	(80 lbs.)	1	
266	31 in.	3	No record of earlier abortions
285	28½ "	1	
298	34 "	1	
301	Alive.	2	Calf killed when 7 days old. Cow aborted in Mar. 1917. (See No. 21.)
313	29 in.	4	No record of former abortions.
316	22¼ "	2	Aborted both times.
342	25¼ "	2	" " "

This group illustrates the fact, already recognized by owners of herds, that cows introduced into an infected herd from a clean herd will abort sooner or later and in this respect act as heifers raised in the herd. They have no protecting immunity. Among the twenty-five cases of Table II are sixteen which may be considered as aborting at the first opportunity; *i.e.*, during what may be called their first pregnancy in the new herd. Seven aborted in the second pregnancy and only two in the third.

Taking together the two groups of 62 cases from which *Bacillus abortus* was isolated either directly or through guinea pigs or with both methods, we find the following classes according to the number of pregnancies: Abortion at first pregnancy, 42; of these 26 were native heifers and 16 purchased cows. Abortion at second pregnancy, 14; of these 7 were native and 7 purchased cows. Abortion, at third pregnancy, 5; 3 were native cows and 2 purchased.

The group of 26 cases from which pure cultures of spirilla have been obtained has been tabulated in other publications.[2,3] Of these only 3 were native cows. The fetuses of these represented the third, sixth, and eighth pregnancy respectively. Among the purchased cows 6 cases were first, 9 second, 5 third, and 3 fourth pregnancies since purchase.

The fourth group of cases from which neither *Bacillus abortus* nor *Vibrio fetus* was isolated forms the most miscellaneous group and one most difficult to analyze. Of the twenty-one cases sufficiently studied through cultures, guinea pig inoculations, and the histological material to deserve a place in the table, one or two may possibly belong to the group of *Bacillus abortus*. Several may come within the group of *Vibrio fetus;* two were cases of infection with *Bacillus pyogenes* in which the preceding pregnancy had been cut short by *Bacillus abortus* infection. In eight, miscellaneous, rapidly growing bacteria appeared in the cultures. Of these several may be regarded as cases in which bacteria gained entrance immediately after expulsion, the fetus being large enough to have lived a short time after birth. One was clearly a case of asphyxiation at birth (No. 126) and one regarded by the

[2] Smith, T., *J. Exp. Med.*, 1918, xxviii, 701.
[3] Smith, T., *J. Exp. Med.*, 1919, xxx, 313.

TABLE II.

Purchased Cows, Aborting or Giving Birth to Living Calves from Which B. abortus Was Isolated.

Case No.	Date of purchase.	Date of calvings after purchase.	Date of abortion.	Size of fetus.	No. of calvings after purchase associated with *B. abortus*.
			1917		
20	Nov. 25, 1915		Mar. 16	Alive.	1
91	Apr. 19, 1917		June 5	30 in.	1
111	Nov. 20, 1915	Sept. 29, 1916	July 16	38 "	2
129		Oct. 2, 1915	Aug. 22	Alive.	2
		" 28, 1916			
131	July 14, 1914	July 9, 1915	" 30	28 in.	3
		Aug. 12, 1916			
			1918		
205	Dec. 14, 1916		Feb. 19	Twins; 35 and 36½ in.	1
214	Apr. 19, 1917	Apr. 24, 1917	Mar. 15	Alive.	1
222	July 25, 1916		Apr. 11	"	1
226	Apr. 3, 1916	Apr. 22, 1917	" 12	"	2
253	Aug. 17, 1917	Oct. 1, 1917	June 13	25 in.	2
270	Oct. 12, 1916	" 16, 1916	Aug. 22	29½ "	2
		Sept. 6, 1917			
273	Nov. 13, 1917	Nov. 20, 1917	" 27	16 "	1
275	Apr. 19, 1917	May 26, 1917	Sept. 2	16¼ "	1
276	Nov. 20, 1915	1917	" 4	20½ "	2
278	" 10, 1917		" 19	14½ "	1
281	" 13, 1917		Oct. 4	30½ "	1
305	Aug. 17, 1917	Sept. 16, 1917	Dec. 24	21½ "	1
			1919		
309	Apr. 6, 1918		Jan. 2	(58 lbs.)	1
311	Aug. 19, 1917		" 7	(38 ")	1
325	Nov. 13, 1917		Feb. 12	36 in.	1
328	Apr. 20, 1917	Aborted Apr. 13, 1918	Mar. 9	31 "	2
329	" 18, 1918		" 11	30 "	1
343	Oct. 12, 1918		Apr. 13	14½ "	1
350	Feb. 9, 1917	Aborted in Nov., 1917, and in May, 1918.	" 30	Alive.	3
351	May 12, 1918	May 25, 1918	May 5	28 in.	1

TABLE III.

Cases of Abortion from Which neither B. abortus nor Vibrio fetus Was Isolated.

Case No.	Length of fetus.	No. of pregnancy.	Purchased or raised.	Date of abortion.	Cultures from.								Remarks.
					Stomach.	Small and large intestines.	Meconium.	Lungs.	Liver.	Spleen.	Kidneys.	Fetal membranes.	
				1916									
4	(8 mos.)	?	Raised.	Apr. 1		—*		—	—		—		No earlier abortions.
35	16½ in.	1	Purchased.	" 14				—	—		—		
				1917									
103	4 "	3	Raised.	June 24								2 g. p. —	No earlier abortions.
122	24 "	1	Purchased.	July 31	Moulds. g. p. —	Moulds. g. p. —			Cocci.	—	—	g. p. —	
126	(75 lbs.)	2	" Sept. 4, 1915.	Aug. 15	— g. p. —	— g. p. —		—	—	—	—	2 g. p. —	Calf probably asphyxiated during birth.
143	(35 ")	1	Purchased Aug., 1917.	Oct. 11	g. p. —			—	—	—	—	2 g. p. —	Cow said to have been fighting few days before abortion.
158	12½ in.	1	Purchased Feb. 16, 1917.	Nov. 6								3 g. p. —	Calved Mar. 10, 1917. Probably a case of spirillum infection.
				1918									
201	34 "	2	Purchased 1917.	Feb. 12	Cultures overgrown.		— 2 g. p. —	Cultures overgrown. 2 g. p. —	—	—	—		Lungs partly inflated.
202	15½ "	4	Purchased Sept. 28, 1917.	" 13	—		— g.p. —	— g. p. —	—	—	—		No record of earlier abortion.

211	23 in.	4	Raised.	Mar. 13	— g. p.—		— g. p.—	—	—	—	—		Preceding calving 2–3 wks. premature (No. 30). *B. abortus* isolated at that time.
259	20 "	2	"	July 18			g. p.—	*B. pyogenes.* g. p.—	*B. pyogenes.*		*B. pyogenes.*		Aborted Oct. 22, 1917 (No. 146).
261	30½ "	6	Purchased Sept. 28, 1912.	" 26	— g. p.—		— g. p.—	— g. p.—	—	—			No record of earlier abortions. Lungs not inflated.
262	29 "	1	Purchased Dec. 5, 1916.	" 28	Strepto-coccus. g. p.—		— g. p.—	Colon bacillus. g. p.—	—	—	Heavy growth.		Lungs not inflated.
265	20 "	4	Raised.	Aug. 4			— g. p.—	Cultures contain *B. coli* types. 2 g. p.—					Calved Feb. 27, 1916. Aborted Aug. 30, 1916. Calved Dec. 20, 1917.
277	32 "	2	"	Sept. 11	Cultures over-grown.	Cultures over-grown.	— g. p.—	Moulds. g. p.—	—	—	—		Premature calf, 7½ mos. Alive. Cow calved normally Aug. 30, 1917.
279	8 "	2	"	" 27				— g. p.—				g. p.—	Aborted Jan. 20, 1918 (first pregnancy). Not studied.
282	Alive.	5	Purchased Aug. 21, 1912.	Oct. 8	Cultures over-grown. g. p.—		— g. p.—	Cultures over-grown. g. p.—					2 wks. premature.

* — indicates negative cultures; g. p. —, negative results with reference to *B. abortus* following the inoculation of guinea pigs.

TABLE III—*Concluded.*

Case No.	Length of fetus.	No. of pregnancy.	Purchased or raised.	Date of abortion.	Cultures from.								Remarks.
					Stomach.	Small and large intestines.	Meconium.	Lungs.	Liver.	Spleen.	Kidneys.	Fetal membranes.	
				1918									
291	32 in.	4	Raised.	Nov. 19	*B. pyogenes.* g. p.—		— g. p.—	*B. pyogenes.* g. p.—	*B. pyogenes.*			g. p.—	No record of previous abortions. Fetus dead some time before expulsion.
300	35 "	6	Purchased Oct. 10, 1911.	Dec. 7	— g. p.—	Stomach—	— g. p.—	— g. p.—	—	—	Cultures overgrown.		Lungs not inflated.
				1919									
327	36 "	1	Purchased Aug. 10, 1917.	Feb. 18	Cultures overgrown. g. p.—		— g. p.—	— One culture overgrown. g. p.—	—	—	—	g. p.—	
344	36½ "	3	Purchased Aug. 20, 1916.	Apr. 12	— g. p.—	Stomach—	—	— g. p.—	—	—	Contaminated.	g. p.—	Calved in 1916 and 1917.

attendants as traceable to fighting several days before expulsion of fetus (No. 143). Seven may be considered bacteria-free or sterile. How many of these are due to injury, to toxic substances, and to food factors cannot be stated. The importance of these cases, because not associated with *Bacillus abortus*, has warranted a rather detailed publication of the data in Table III.

In this group too there are no first pregnancies. Those marked first in the table refer to purchased cows and are at least second pregnancies. Thirteen are purchased and eight native cows. The pregnancies during which the fetuses were dropped prematurely range from first to sixth.

Summing up the bacteriological data from another point of view we have 47 cases from which *Bacillus abortus* was not isolated. Among the latter were 25 cases associated with *Vibrio fetus* and 1 case containing a related but not identical spirillum; 2 with *Bacillus pyogenes*; 7 with sterile digestive and respiratory tracts; and 7 with only cultures of the digestive tract and lungs fertile, due chiefly to aspiration and swallowing of miscellaneous bacteria at birth.

Acquired Immunity to Bacillus abortus.

A statement frequently made in veterinary literature and by stock owners is that cows gradually lose the tendency to abort. This tendency is in harmony with the nature of the abortion disease,—an invasion of the fetal membranes, more particularly the chorion, by infectious agents, followed by multiplication of the latter and more or less injury to the tissues attacked.

The rapidity with which immunity is acquired varies considerably. Holth[4] quotes one observer as giving the experience of thirty veterinarians to the effect that cows tend to abort two or three times in succession. He quotes another observer as claiming that 68 per cent of aborting cows aborted but once, and Bang as stating that of 83 aborting heifers only 20 aborted next time. In most infectious diseases the young are attacked in largest numbers. Hence we would expect the largest number of abortions among primiparæ (heifers). But even here observations differ. Holth quotes an observer as

[4] Holth, H., *Z. Infektionskrankh. Haustiere*, 1911, x, 342.

stating that only 36 per cent of the cows under his care calved normally, whereas 45 per cent of the heifers did so.

In the tables it is evident that abortion is essentially a disease of young stock and that second and third abortions are less common. If we control the data by the bacteriological results obtained, we find that relatively few cows are subject to disease of the fetal membranes due to *Bacillus abortus* twice. Abortions do occur in succession but they are due chiefly to causes other than *Bacillus abortus*. In illustration of this fact the following consecutive abortions studied bacteriologically are noteworthy. The individual abortions have been given case numbers; each pair of numbers therefore refers to one and the same cow.

Case 3.—Cow aborted Feb. 23, 1916. Had six or seven normal calves before. Fetus contained within membranes, about 6 to 7 inches long. Fetus has undergone autolytic changes. Only head, limbs, and ribs recognizable. The entire mass has a reddish, translucent, gelatinous appearance. Guinea pigs inoculated with suspensions of ground cotyledons remained well. Cultures from spleens negative. This case cannot be considered as having been examined thoroughly bacteriologically, but *B. abortus* was most probably absent.

Case 159.—The same cow aborted Nov. 7, 1917, after having calved normally Feb. 12, 1917. Fetus 16¾ inches long. Some of the placenta discharged. Fetus had been dead *in utero*, probably for some time. From this case spirilla were isolated. Tests for *B. abortus* negative.

Case 146.—Holstein heifer aborted on pasture Oct. 22, 1917. Fetus could not be found. A portion of placenta removed next day. All of twelve guinea pigs inoculated from this for another experiment were found infected with *B. abortus* when chloroformed. *B. abortus* also identified in sections of placenta.

Case 259.—The same cow aborted July 18, 1918. Fetus 20 inches long. General subcutaneous, blood-tinted edema. Some of abdominal organs eaten out by some animal. Thorax full of blood-tinted fluid. Fetus probably dead some time as autolytic changes were prominent. Cultures from intact organs—lungs, liver, and kidneys—show large numbers of colonies of a bacillus diagnosed as *B. pyogenes*. Guinea pig tests of lung and meconium negative.

Case 30.—Calved Apr. 3, 1917, but calf lived 2 hours only. Probably 2 weeks premature. This cow had calved normally in 1914 and 1916. *B. abortus* was isolated from the uninflated lungs both in cultures and through guinea pigs.

Case 211.—The same cow aborted Mar. 13, 1918. Placenta retained. Fetus 23 inches long; has undergone more or less maceration *in utero*. Cuticle peels off readily. Large serous cavities filled with blood-tinted fluid. Edema of subcutis and muscular tissue, blood-stained. Cultures from this case remained sterile. Guinea pig tests also negative.

Case 19.—Cow gave birth to twin calves Mar. 13, 1917 (second pregnancy). Both very weak, unable to stand and too weak to drink. One calf died Mar. 15, of scours. Extensive fatty degeneration of liver. Many collapsed lobules throughout left lung and in ventral lobe of right lung. Two guinea pigs inoculated with suspension of ground lung tissue in salt solution became infected with *B. abortus*.

Case 290.—The same cow on Nov. 19, 1918, gave birth to a fetus 24 inches long. Placenta retained. Guinea pig inoculations negative as to *B. abortus*. Spirilla in pure culture isolated from fourth stomach and colon contents, from liver, spleen, kidney, and lungs.

Case 21.—Heifer aborted Mar. 20, 1917. Fetus about 25 inches long. General subcutaneous edema. Large serous cavities filled with blood-tinted fluid. From the placenta *B. abortus* was isolated through a guinea pig.

Case 301.—The same heifer was bred four times and gave birth on Dec. 11, 1918, to a calf apparently normal. Placenta retained; cow was discharging from vagina. A swab containing such discharge washed out in salt solution and the latter injected into two guinea pigs. From both *B. abortus* was subsequently isolated. The calf did not do well and was killed when 7 days old. One umbilical artery close to umbilicus was involved in suppuration. Multiple foci of collapse in both ventral lobes. From a guinea pig inoculated therewith *B. abortus* was isolated.

Case 246.—Fetus 7 to 8 inches long found enclosed in membranes on the morning of May 28, 1918. Autolytic changes far advanced. No odor of bacterial decomposition. Spirilla were isolated in pure culture from the lungs and liver. No other bacteria found in the fetus. Guinea pigs inoculated with material from the same organs were normal when chloroformed after 6 weeks. This cow was purchased Sept. 2, 1917, and aborted in Nov. of the same year. The fetus from this abortion was not received for study.

Case 350.—The same cow aborted a third time Apr. 30, 1919. Fetus, due June 4, alive at birth and able to stand. Weighs 57 lbs. Killed about 4 hours after birth. Autopsy shows that no food had been taken but the rumen contained besides the fetal fluid much froth from swallowed saliva. No abnormalities were detected. The placenta was retained and films from a swab inserted into uterus showed groups of bacteria resembling *B. abortus*. Cultures from the stomachs were impure mixtures, as might have been expected, but those from colon and rectum contained only *B. abortus*. A guinea pig inoculated with salt solution suspension from the swab, chloroformed after 7 weeks, was found with the lesions of *B. abortus*.

These six pairs of consecutive abortions indicate in general an acquired immunity to *Bacillus abortus*. In three pairs the first abortion was associated with *Bacillus abortus*, the second with negative cul-

tures, *Bacillus pyogenes*, and spirilla respectively. In one pair both abortions were associated with *Bacillus abortus*. In one pair *Bacillus abortus* was not detected either time. The first abortion may or may not have been associated with spirilla, since the condition of the material (advanced autolysis) did not warrant cultures, except from the spleen. These were negative. Later studies indicated that the spleen is rarely invaded by spirilla. The last case is exceptional. Of the first abortion there is no record. It may or may not have been due to *Bacillus abortus*. The second abortion was associated with spirilla. The third, associated with *Bacillus abortus*, may have been due to reinfection from the udder following loss of immunity acquired on account of the first abortion.

Were it not for agents other than *Bacillus abortus*, such as *Vibrio fetus*, and to a far less degree to miscellaneous septic and pyogenic organisms, and to unknown, non-bacterial agencies, second and later abortions by the same cow would be relatively rare. It therefore becomes necessary in the future to distinguish between the tendency of any given cow to abort repeatedly and the relation of *Bacillus abortus* to such repeated abortions. If abortions are due to a variety of infectious and non-infectious agencies, a better knowledge of abortion can only be reached by a patient, thorough study of series of individual cases, followed by the necessary experimental tests in order that these agencies may be assigned their proper place. A further analysis of this material will be found elsewhere.[5] It is there shown that the udders of a relatively high percentage of cows become infected with *Bacillus abortus* probably during the first abortion disease. Cows while carrying *Bacillus abortus* in the udder may give birth to normal calves, or to fetuses infected with spirilla, or to sterile fetuses. Probably the udder becomes a protecting reservoir of immune bodies towards *Bacillus abortus*.

SUMMARY.

In a large herd of dairy cattle and young stock the bacteriological examination of 109 cases of abortion which included a relatively thorough study of the fetus and a study of the membranes, or swabs from

[5] Smillie, E. W., Little, R. B., and Florence, L., *J. Exp. Med.*, 1919, **xxx**, 341.

the uterus whenever obtainable, gave the following results. 62, or 57 per cent, were associated with *Bacillus abortus*. 26, or 23.8 per cent, were associated with spirilla. 2, or 1.8 per cent, were associated with *Bacillus pyogenes*. 19, or 17.4 per cent, were either sterile or else the digestive and respiratory tracts had been invaded during or after birth with miscellaneous bacteria. *Bacillus abortus* was absent according to cultures and animal tests.

Such a relatively large proportion of cases of abortion without *Bacillus abortus* as the inciting agent is noteworthy. In general *Bacillus abortus* was associated with first pregnancies. Its presence diminished rapidly in frequency in later pregnancies. Assuming in a general way that purchased cows coming from small herds were free from any immunity and that their first pregnancy in the new herd is equivalent to that of a native heifer and may be counted as the first, we have *Bacillus abortus* associated with the first pregnancy in 42, with the second in 14, with the third in 5, and with the fourth in 1. Spirilla were distributed as follows: (*a*) in purchased cows, first pregnancy, 6; second pregnancy, 9; third pregnancy, 5; and fourth pregnancy, 3; (*b*) in native cows, first pregnancy, 0; third pregnancy, 1; sixth pregnancy, 1; and eighth pregnancy, 1. The relation of infection with spirilla to acquired immunity is not clear and more data from large herds are needed to define both etiological and immunological bearings of the spirilla.

Thus far spirilla have not been encountered in native heifers of the herd giving birth the first time. A tentative explanation to be offered is that the young stock is kept segregated from the older and purchased cows until shortly before calving. The occasional discharge of a fetus among the young stock in pasture tends to keep up the disease due to *Bacillus abortus*. Later on association with older cows brings about infection with spirilla (*Vibrio fetus*) and more rarely with other possible agencies of fetal disease. On the other hand, abortions may occur among the pastured stock from time to time and remain unrecognized. Not until both groups of animals are subjected to the same daily scrutiny will it be possible to affirm that abortion associated with spirilla does or does not occur among young stock.

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AN INTERPRETATION OF THE AGGLUTINATION REACTION TO BACILLUS ABORTUS IN 75 CASES OF BOVINE ABORTION BACTERIOLOGICALLY CONTROLLED.

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In the following pages are given the results of a study of the agglutination test in 75 cases of abortion of which bacteriological data were at hand. Concerning these data and the methods employed in obtaining the results here used to interpret our findings, the reader is referred to Dr. Smith's paper.[1] Although bacteriological data on a much larger number of cases were available, yet in many instances the cows were removed from the herd before samples of blood could be taken. While this work was in progress certain discrepancies came to the surface and the search for a focus of infection with *Bacillus abortus* in the udder was deemed essential by Dr. Smith. Accordingly the milk from certain selected cases was brought into the investigation.

The existing literature on the agglutination test is voluminous and the work in most cases of high quality, yet there is lacking the bacteriological basis of a series of spontaneous cases for a more accurate interpretation of the test. The fact that Smith[1] found in 109 cases of abortion or premature expulsion of a living calf 46 not associated with *B. abortus* shows that our information in regard to the significance of the agglutination test has been built on somewhat insecure foundations.

Most writers have confined their attention to a comparison between the agglutination and the complement fixation test. This was done by Mohler and

[1] Smith, T., *J. Exp. Med.*, 1919, xxx, 325.

Traum,[2] Moore and Fitch,[3] Larson,[4] Rettger and White,[5] Wall,[6] and Zwick and Zeller.[7] Others have contented themselves with associating the serological reactions with abortion pure and simple. Wall,[6] Grinsted,[8] and Moore and Fitch[3] studied among other things the rise and decline of the agglutination curve. Since none of these researches bears directly upon the substance of this communication any detailed analysis would be out of place.

Methods.

Agglutination Technique.—The obtaining of blood samples from cattle is frequently difficult, since it depends on the way the animals are stabled and their degree of wildness or domestication. Those from which samples were collected for the present investigation were in the main milking cows. They were secured in stanchions, facing on a wide alleyway. A rope halter was placed on the head of the animal and the neck and head were extended by fastening the rope, so attached, to an iron rail supporting stanchions on the other side of the alley, and tightening the rope gradually until the jugular furrows were fully exposed. Strong bull nose forceps were then put on the anterior part of the nasal septum to hold the attention of the animal, and also to steady the head. One assistant can handle both the rope and bull nose forceps.

When the animal was well under control the field of operation was thoroughly wiped off with 95 per cent alcohol. Pressure was applied over the jugular vein at the posterior end of the furrow to make the vein prominent and a $2\frac{1}{2}$ inch needle No. 14 was inserted. 25 to 30 cc. of blood were collected in a sterile 50 cc. centrifuge tube. A separate, sterile needle was used for each sample. The samples were stored at room temperature until the clot had formed, after which it was separated from the sides of the tube with a heavy sterile wire.

[2] Mohler, J. R., and Traum, J., *U. S. Dept. Agric., Bureau of Animal Industry, Bull. 216*, 1913, 147.

[3] Moore, V. A., and Fitch, C. P., *Rep. N. Y. State Vet. College*, 1912–13, 82.

[4] Larson, W. P., *J. Infect. Dis.*, 1912, x, 178.

[5] Rettger, L. F., and White, G. C., *Storrs Agric. Exp. Station, Bull. 93*, 1918.

[6] Wall, S., *Z. Infektionskrankh. Haustiere*, 1911, x, 23, 132.

[7] Zwick and Zeller, *Arb. k. Gsndhtsamte.*, 1912, xliii, 1.

[8] Grinsted, P., Maanedsskrift f. Dyrlaeger, 1909, xxi, Review in *Berl. tierärztl. Woch.*, 1909, xxv, 831.

The blood samples were then placed in the refrigerator for 12 hours to allow the clot to contract completely. Each tube was centrifuged for 10 minutes, to throw the clot and free corpuscles to the bottom. With sterile bulb pipettes the clear serum was transferred from the centrifuge tubes to sterile, corked test-tubes for storage. No disinfectant was added to the serum as it was found unnecessary if each step of the procedure was carried out carefully.

The antigen used was always prepared fresh as needed. A stock culture of *Bacillus abortus* recovered early in this work by Dr. Smith was used. A 48 hour slant agar culture was washed off with 2 cc. of normal saline solution and the suspension transferred to an agar surface within a rectangular 16 ounce bottle. The bottle was sealed and incubated for 72 hours at 37°C., then washed off with 20 cc. of normal saline solution, and the suspension transferred to a sterile bottle. This very dense suspension was standardized by reducing the density with normal saline solution to a point at which the translucency equals that of a 24 hour bouillon culture of the typhoid bacillus. It was then ready for use.

In order to simplify the technique as much as possible, reduce the amount of glassware used, and safeguard the operations by using the same amounts of fluids in the series of tubes in which clumping was to be observed the following method was used throughout. Eight stock dilutions of serum were used as a starting-point. To the first tube 4 cc. of salt solution and 1 cc. of undiluted serum were added to make a stock dilution of 1:5. Into each of the other tubes $2\frac{1}{2}$ cc. of salt solution were placed. Then $2\frac{1}{2}$ cc. of the 1:5 serum dilution were added to the second tube, the mixture was drawn up several times, and $2\frac{1}{2}$ cc. of this were transferred to a third tube, and so on successively to the last. Each tube, except the final dilution, then contained $2\frac{1}{2}$ cc. of salt solution and $2\frac{1}{2}$ cc. of serum dilution, making 5 cc. in all and furnishing dilutions of serum beginning with 1:5 and ending in 1:640. To eight fresh tubes $\frac{1}{2}$ cc. of each serum dilution was now added, beginning with the highest dilution and using the same pipette up to the most concentrated solution. Finally $\frac{1}{2}$ cc. of the suspension of bacteria was added, making a series of serum dilutions beginning with 1:10 as shown in Table I. Particles in the culture suspension, such as bits of agar and clumps of bacteria, were

removed by withdrawing the suspension with a pipette through a piece of sterile cotton dropped into the stock suspension.

The distance or interval between the last tube of the series which was completely cleared by the clumping and sedimentation and the tube which showed the agglutination limit varied somewhat from case to case, as shown in Table II, which contains three illustrative examples taken from the protocols.

TABLE I.

Serum Dilution. (*First Series of Tubes.*)

Tube No.	1	2	3	4	5	6	7	8
	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
Salt solution added	4–	2½–	2½–	2½–	2½–	2½–	2½–	2½
Undiluted serum to first tube	1–							
Diluted serum to other tubes in succession		→2½–	→2½	→2½–	→2½–	→2½–	→2½–	→2½
Dilutions	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640.

Final Dilution. (*Second Series of Tubes.*)

Serum dilutions	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640
	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
Amount of serum dilution added to each tube	½	½	½	½	½	½	½	½
Culture suspension	½	½	½	½	½	½	½	½
Final dilution	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280

This method of using at least eight final dilutions served to reveal any possible errors and control any so called paradoxical reactions. Unless the series showed a definite regular decline in agglutinins, it was done over again. By this means most paradoxical reactions were traced to some irregularity or accidental error in manipulation. Table II illustrates the protocols. Readings were made after 3 to 4 hours in the incubator, followed by refrigeration over night.

The final agglutination titer adopted was shown by that tube in which there was some definite clumping as indicated in the suspension

or amount of deposit when compared with the next lower and negative tube and the culture control. In Table II the limit is shown as 1:640 in Case 31. Nos. 19 and 101 require further dilutions to obtain the precise limit. When clumping occurred in the control the series was rejected and a fresh one prepared. The figures given in Tables III to V represent, therefore, the agglutination limit under the conditions of the test as described above.

Test for Bacillus abortus in the Udder.—The milk was collected directly into sterile glass jars during the latter part of milking. The samples averaged about 650 cc. At the laboratory the milk was transferred to sterile separatory funnels and kept over night at

TABLE II.

Illustration of the Range between Complete Sedimentation and Slight or No Clumping.

Case No.	Dilutions.								Control.
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	
31	C.*	C.	C.	C.	++ ++	++ +	++	−	−
101	"	"	"	"	C.	++ ++	++ ++	++	−
19	"	"	"	"	"	C.	C.	++ +	−

* C. indicates complete clearing of the fluid, ++++ slight clouding plus heavy deposit, and so on to −, which means no trace of clumping.

about 4–5°C. The next morning the milk was withdrawn from the funnel, leaving the cream, distributed into large centrifuge bottles, and centrifuged for 20 minutes at a speed of about 1,500 revolutions per minute. Any cream remaining was removed with a section lifter, some of the top milk withdrawn with a pipette or syphon, and the remaining milk and sediment were well mixed and distributed into smaller centrifuge tubes and centrifuged a second time for 15 minutes at a speed of about 1,400 revolutions per minute. The top milk was again withdrawn, the remaining milk and sediment were well mixed, and each sample was injected intraperitoneally into three guinea pigs. Each animal received 5 to 7 cc. Films of the milk

fresh and fixed in absolute alcohol and ether and stained with alkaline methylene blue or Giemsa's stain were examined to determine in general the character of the sediment injected. After a period of 6 to 8 weeks the guinea pigs were chloroformed and three cultures made from each animal by tearing out bits of spleen tissue and transferring to agar slants whether lesions were present or not. All cultures were sealed.

Agglutination Titer towards Bacillus abortus when the Fetus Contains Only Spirilla (Vibrio fetus).

For more detailed information concerning the bacteriological data used in the tables to follow, the reader is referred to earlier papers by Smith.[1,9,10] Certain unpublished data were also furnished by him in individual cases to be discussed later.

In Table III are given data pertaining to nineteen cases in which spirilla were found and agglutination tests made. The remaining seven cases of the same group are omitted because blood samples were not obtained.

Taking an agglutination titer up to and including 1:40 as negative as to the presence of *Bacillus abortus*, we find that in eleven cases the agglutination titer agrees with the bacteriological findings. Of these eleven cases, the milk of three was examined for *Bacillus abortus* and found free. In two cases (Nos. 256 and 290) a maximum titer of 1:1,280 for *Bacillus abortus* was associated with the presence of this bacillus in the milk. This high titer is thus accounted for. The first four cases in the table have titers which indicate that the animals were infected with *Bacillus abortus* at some earlier date, since in three of these the milk tests were negative. The fourth was not examined. Of these No. 246 is of interest. This cow aborted May 28, 1918, with spirilla in the fetus. The fetus was small, only $7\frac{1}{2}$ inches long. On September 5 and 12, blood samples registered a titer of 1:160. On February 4, 1919, a sample of milk proved free from *Bacillus abortus*. On April 30, the cow aborted again (now Case 350). *Bacillus abortus* was found in the fetus. The titer of the

[9] Smith, T., *J. Exp. Med.*, 1918, xxviii, 701.
[10] Smith, T., *J. Exp. Med.*, 1919, xxx, 313.

blood on May 16 was 1:640. Evidently a fresh infection with *Bacillus abortus* had taken place since the previous abortion. In No 159 there was a decline in the agglutination titer following the birth of a normal calf, asphyxiated at birth. Case 251 is the only one in the table whose titer cannot be satisfactorily explained with the data on hand. This cow aborted June 10, 1918, with positive spirilla cultures. The results were negative as to *Bacillus abortus*. On September 5, the blood titer for *Bacillus abortus* was 1:640. Two milk tests with samples, collected November 20, 1918, and March 4, 1919, were both negative. The only explanation that suggests itself is that there was associated *Bacillus abortus* with the spirilla in the fetal membranes but not in the fetus in the June abortion. The absence of any tests with uterine exudate immediately after abortion leaves this interpretation open. It is also possible that *Bacillus abortus* was in the milk in very small numbers. This case is thus the only one in the table in which the serological test does not harmonize with the bacteriological data.

One of the most instructive cases in Table III is Cow 290. The peculiarity of the agglutination titer makes a more detailed history of this case of interest. This cow gave birth to twins March 13, 1917. Both were unable to drink and too weak to stand. One was brought to the laboratory dead March 15. From Dr. Smith's notes on this case we find that there was marked fatty degeneration of the liver and extreme congestion of the medullary zone of the kidneys. The mucosa of the fourth stomach was covered with a thick viscid layer of mucus. In the lungs there were many lobules and groups of lobules collapsed. The postmortem changes made examination of the small intestines useless but the calf probably died of what is usually known as scours. Histological examination of fixed and hardened tissues showed bronchopneumonic collections of cells resembling polynuclear leucocytes, general vacuolation of liver cells, and intense congestion of the medulla of the kidneys approaching hemorrhage. Pieces of the collapsed lung tissue ground and inoculated into two guinea pigs produced the abortion disease in both cases. From these *Bacillus abortus* was isolated.

A dead fetus was discharged November 19, 1918, 24 inches long (No. 290). Spirilla were isolated in pure culture from the fourth

TABLE III.

Agglutination Test for B. abortus in Cases of Abortion Associated with Spirilla.

Case No.	Length of fetus.	Native or purchased cow.	Date of abortion.	No. of pregnancy.	Date of blood sample.	Agglutination titer (limit).	Date of milk sample.	Test for *B. abortus* in milk.	Remarks.
	in.								
159	16¾	Purchased.	Dec. 17, 1917	3	Aug. 29, 1918	1:160			Preceding pregnancy also terminated in abortion. Etiology in doubt Asphyxiated at birth.
(324)	Normal calf.		Feb. 12, 1919	4	Mar. 24, 1919	1:20			
192	36	Purchased.	Jan. 23, 1918	3	Aug. 29, 1918	1:80	Dec. 17, 1918	−	
213	21	"	Mar. 18, 1918	4	" 29, 1918	1:160	" 17, 1918	−	
246	7½	"	May 28, 1918	2	Sept. 5, 1918	1:160	Feb. 4, 1919	−	
					" 12, 1918	1:160			
(350)	Calf alive.		Apr. 30, 1919	3	May 16, 1919	1:640			*B. abortus* found in calf.
251	17½	Purchased.	June 10, 1918	2	Sept. 5, 1918	1:640	Nov. 20, 1918	−	
							Mar. 4, 1919	−	
256	19	"	" 18, 1918	4	Oct. 3, 1918	1:1,280	Nov. 27, 1918	+	
258	27	"	" 29, 1918	2	" 3, 1918	1:20	Feb. 11, 1919	−	
267	23	"	Aug. 12, 1918	3	" 3, 1918	1:40			
269	20	"	Nov. 18, 1918	2	Nov. 21, 1918	1:20	Jan. 7, 1919	−	
					" 28, 1918	1:20			

290	24	Native.	Nov. 19, 1918	3	Nov. 14, 1918 " 21, 1918 " 28, 1918 Dec. 11, 1918	1:10 1:1,280 1:1,280 1:1,280	Nov 27. 1918	+	Preceding pregnancy (twins) associated with *B. abortus* (Mar. 13, 1917). Agglutination titer still 1:1,280 Apr. 4, 1919.
308	31½	Purchased.	Dec. 30, 1918	1	Jan. 2, 1919	1:40			
317	25	"	Jan. 27, 1919	2	Feb. 7, 1919	1:10	Mar. 21, 1919	−	
318	26	"	" 27, 1919	2	" 7, 1919	1:10			
331	8	Native.	Mar. 15, 1919	6	Mar. 24, 1919	1:10			
333	26	Purchased.	" 27, 1919	1	" 29, 1919	1:10			
339	35½	"	Apr. 9, 1919	3	" 29, 1919	1:40			
351	28	"	May 5, 1919	1	May 15, 1919 June 2, 1919	1:20 1:160			Fetus contained *B. abortus* also.
356	26½	"	" 23, 1919	1	" 2, 1919	1:80			
357	21¾	Native.	" 24, 1919	8	" 2, 1919	1:10			

stomach, large intestine, lungs, kidney, spleen, and liver. Guinea pigs inoculated with lung tissue, meconium, fourth stomach contents, and suspensions of exudate from the vagina obtained with a swab November 18 were normal when killed after 7 weeks.

On November 14, 1918, 5 days before abortion, the agglutination titer was only 1:10. 7 days later and 2 days after discharge of the fetus it was 1:1,280. The same high titer was found on November 28 and December 11. Milk drawn November 27 was found infected with *Bacillus abortus*. On April 4, 1919, the titer was still 1:1,280.

The main facts in the history of this cow are: Twins March 13, 1917, one of which carried *Bacillus abortus* in lungs. Discharge of fetus November 19, 1918, containing spirilla but not *Bacillus abortus*. Agglutination titer towards *Bacillus abortus* jumped from 1:10, 5 days before, to 1:1,280, 2 days after abortion. We have thus far been unable to find a second case with conditions paralleling this and hence are unable to present a confirmation of this somewhat unique case. Possible errors in collecting samples and in assigning the fetus to the right dam can be eliminated. The sudden rise in agglutinins may be tentatively ascribed to a rapid absorption of *Bacillus abortus* or some antigen from the udder into the circulation during discharge of the fetus. The mechanism for producing agglutinins towards *Bacillus abortus* having been established since the preceding calving, it acted promptly on stimulation by discharging large amounts of agglutinin into the blood.

No. 356 has a suspicious titer probably due to earlier *Bacillus abortus* infection. No. 351, a double infection, is discussed farther on.

In Table IV are brought together such cases of abortion as were characterized by sterile fetuses or else by miscellaneous infection of the digestive and respiratory tracts. Bacteriological details will be found elsewhere.[1]

Of the eleven cases, eight show a titer of 1:20 or lower. One (No. 211) with a high titer of 1:640 is explained by an earlier *Bacillus abortus* infection and by a demonstration of *Bacillus abortus* in the milk. The first sample of milk was negative, the second positive. In another (No. 259) with a titer of 1:160 this may be regarded as a remnant of the preceding pregnancy which terminated in abortion associated with *Bacillus abortus*. The same may be true of a third

TABLE IV.

Agglutination Titer in Cases of Abortion with Sterile Fetus or Miscellaneous Infection.

Case No.	Length of fetus.	Native or purchased cow.	Date of abortion.	No. of pregnancy.	Date of blood sample.	Agglutination titer (limit).	Date of milk sample.	Test for *B. abortus* in milk.	Remarks.
	in.		*1917*		*1918*		*1919*		
103	4	Native.	June 24	3	Oct. 30	1:10			Normal births Aug. 8, 1918, and Mar. 26, 1919.
122	24	Purchased.	July 31	1	Nov. 14	0			Normal calf Nov. 16, 1918.
126	(75 lbs.)	"	Aug. 15	2	" 14	1:20			" " " 19, 1918.
143	(35 ")	"	Oct. 11	1	Oct. 30	1:20	Jan. 10	—	" " Oct. 17, 1918.
			1918						
201	34	"	Feb. 12	2	" 9	1:10	" 10	—	
211	23	Native.	Mar. 13	4	Sept. 27	1:640	" 10	—	Preceding pregnancy terminated Apr. 4, 1917, by *B. abortus* infection.
					1919				
					Apr. 26	1:1,280	Apr. 11	+	Normal calf Apr. 4, 1919.
					June 3	1:2,560			
					1918				
259	20	"	July 18	2	Oct. 3	1:160	Jan. 2	—	Preceding pregnancy infected with *B. abortus*; present one with *B. pyogenes*.
262	29	Purchased.	" 28	1	" 3	1:20			
279	8	Native.	Sept. 27	2	" 3	1:320			Aborted Jan. 20, 1918. No bacteriological data.
291	32	"	Nov 19	4	Nov. 28	1:10	Jan. 2	—	Infection with *B. pyogenes*.
300	35	Purchased.	Dec. 7	6	Dec. 11	1:20			

TABLE V.

Agglutination Reaction of Cows, Native and Purchased, from Which B. abortus Was Isolated.

Case No.	Length of fetus.	Native or purchased cow.	Date of abortion.	No. of pregnancy.	Date of blood sample.	Agglutination titer (limit).	Date of milk sample.	Test for *B. abortus* in milk.	Remarks.
	in.		*1917*		*1918*		*1918*		
17	27	Native.	Mar. 9	1	Nov. 7	1:40			Normal calf Apr. 14, 1918.
26		"	" 27	1	" 21	1:20			" calves Jan. 17, 1918, and Mar. 22, 1919.
31	27	Purchased.	Apr. 5	1	" 28	1:640	Dec. 17 *1919*	+	Normal calf Oct. 3, 1918.
33	26	Native.	" 6	1	" 21	1:640	Feb. 18	+	" " June 8, 1918.
34*	Living calf.	"	Feb. 23		" 21	1:320	" 20	+	" calves Feb. 23, 1917, and Mar. 3, 1918. Cow aborted Nov. 10, 1918. No bacteriological examination.
41	Nearly mature.	"	Apr. 22	1	" 14	1:40	" 20	+	Normal calf Oct. 17, 1918.
43	(8 mos.)	"	" 23	1	" 21	1:640	" 20	+	" " Apr. 23, 1918.
86	21½	"	May 16	1	" 14	0			" " Oct. 25, 1918.
89	Alive.	"	" 16	1	" 21	1:40			" " Aug. 6, 1918.
91	30	Purchased.	" 5	1	" 21	1:80			" " " 1, 1918.
101*	Normal calf.	Native.	June 28	1	Oct. 30	1:1,280	Feb. 28	+	Placenta appears normal. Normal calf June 9, 1918.
128	Calf dies (scours).	Purchased.	Aug. 17	1	" 20	1:10			*B. abortus* isolated from calf's intestine. Normal calf Sept. 26, 1918.
134	Calf alive.	Native.	Sept. 4	1	Nov. 14	1:320	Feb. 18	+	*B. abortus* obtained from placenta. Normal calf Nov. 8, 1918.

144*	Normal calf.	Purchased.	Oct. 8	1	Oct. 30	1:640	Mar. 4	+	Normal calf Mar 21. 1919.
164	Alive.	Native.	Nov. 20	1	" 9 *1919*	1:320	" 4	+	7 wks. premature.
188*	Calf alive.	Purchased.	Dec. 31 *1918*	1	Jan. 2 *1918*	1:1,280	Feb. 26	−	
200	21	Native.	Feb. 12	1	Oct. 17	1:640	" 26	+	Calf very sick with scours when killed.
203	Calf alive.	"	" 15	1	Sept. 27	1:640			
206	28	"	" 24	1	" 27	1:160			
210	Calf alive.	"	Mar. 6	1	" 27	1:40			
214	" "	Purchased.	" 15	1	" 27	1:320			
215	" "	Native.	" 11	1	" 18	1:1,280			
220*	" "	"	" 30	1	" 18	1:640	*1918* Nov. 12	+	Multiple staphylococcus abscesses in lung.
222	" "	Purchased.	Apr. 11	1	" 18	1:1,280			
224	" "	Native.	" 12	1	" 18	1:640			
226	" "	"	" 12	2	" 12	1:1,280			
227	" "	"	" 15	1	" 12	1:640			
253	25	Purchased.	June 13	2	" 27	1:160			
					Oct. 3	1:320			
266	31	Native.	Aug. 5	3	Sept. 5	1:640	Nov. 20 *1919*	+	
270	29½	Purchased.	" 22	2	" 5	1:1,280	Jan. 11	−	
273	16	"	" 27	2	" 5	1:320			
278	14½	"	Sept. 19	1	Oct. 4	1:40			
281	30½	"	Oct. 4	1	" 9 *1919*	1:160			
285	28½	Native.	" 23	1	Jan. 2	1:640			
288*	Calf alive.	Purchased.	Nov. 17	1	" 2	1:1,280	Feb. 4	+	Calf has scours

* Included because the milk was examined. *B. abortus* was not isolated from the calf.

TABLE V—*Concluded.*

Case No.	Length of fetus.	Native or purchased cow.	Date of abortion.	No. of pregnancy.	Date of blood sample.	Agglutination titer (limit).	Date of milk sample.	Test for *B. abortus* in milk.	Remarks.
	in.		*1918*		*1918*		*1918*		
298	34	Native.	Dec. 2	1	Dec. 11	1:80		-	
301	Calf alive.	"	" 11	2	Nov. 28	1:1,280	Dec. 10	+	*B. abortus* isolated from uterine exudate. *B. abortus* found in fetus expelled Mar. 20, 1917.
			1919		*1919*				
309	(58 lbs.)	Purchased.	Jan. 2	1	Jan. 8	1:320			
311	(38 ")	"	" 7	1	" 16	1:640			
313	29	Native.	" 12	4	" 23	1:640			No record of former abortions.
316	22¼	"	" 18	2	" 18	1:1,280			
328	31	"	Mar. 9	2	Mar. 24	1:640			
329	30	Purchased.	" 11	1	" 24	1:640			
350	Calf alive.	"	Apr. 30	3	May 16	1:640			Aborted in preceding pregnancy.
351	28	"	May 5	1	" 15	1:20			Fetus contains both spirilla and *B. abortus*.
					June 2	1:160			

case (No. 279). Unfortunately a milk sample from this cow was not obtainable.

In Table V all cases of abortion associated with *Bacillus abortus* in the fetus or membranes or in the udder are brought together. The agglutination limits as given easily bring the cases into several categories. Taking those cases in which the serum clumps *Bacillus abortus* in dilutions of 1:20 and 1:40, but not up to 1:80, we find, out of 44, 8 in this class. The low titer in certain cases (Nos. 17, 26, 41, 86, 89, and 128) may be referred to the long period between abortion and the blood test, ranging from 14 to 20 months. In No. 210, the low titer of 1:40 may be due to a mild, possibly quite recent infection of the fetal membranes. The calf was vigorous and apparently in normal condition. No. 278 with a titer of 1:40 discharged a macerated fetus about 3½ months old. Here also the infection may have been too recent to stimulate antibodies.

No. 351 is of interest since both *Vibrio fetus* and *Bacillus abortus* were isolated from the fetus. The dam was well along (6½ to 7 months) in pregnancy. *Bacillus abortus* infection may have been superinduced late on the spirillum infection, since the agglutination titer for *Bacillus abortus* rose after abortion.

There are but two cases with a titer of 1:80. In one (No. 91) this was determined 18 months after abortion and may have fallen from a higher level. The second (No. 298) cannot be explained in the same way since the blood test was made 9 days after abortion. In this case, however, the fetus was large and the duration of pregnancy over 7 months. Here also the immune reaction of the dam may not have reached its maximum owing to late infection.

The two cases with a titer of 1:160 are probably explainable in much the same way as the two preceding ones. In one the blood test was made 5 days, in the other 7 months after abortion. Both fetuses were large.

Taking the next higher titer, 1:320, we find six on this level. Nos. 134 and 164 carried *Bacillus abortus* in the milk according to recent tests. How far back this milk infection dates cannot be determined. Most likely it started with the abortion indicated in the table. In the case of Nos. 214, 253, 273, and 309 the longest period between the discharge of fetus or calf and the agglutination test was less than 7 months.

The next highest titer, 1:640, was held by sixteen cases. In the two earliest cases recorded (Nos. 31 and 33) the blood test followed abortion by 19 months. The titer should have been low after such a long period but the udder was infected. In the next case (No. 144) the blood test followed abortion by nearly 13 months. In this case also the high titer is explainable by the udder infection. In the remaining thirteen cases the high titer is amply accounted for by the recent abortion. The milk, tested in only three of these, was found infected in all.

The highest recorded titer in this group, 1:1,280, was held by nine cases. The agglutination test in No. 101 is referable to the milk infection. No. 188 is an unexplained case. The test for *Bacillus abortus* in the milk was negative. A second sample is now being examined.[11] The other cases may be explained by the presence of *Bacillus abortus* in the fetus. No. 270 is of interest in that a high titer was present 14 days after abortion, although the sample of milk tested subsequently was free from infection.

SUMMARY.

The agglutination test when carried out so as to give the entire range of serum dilutions to the limit of clumping is a delicate test which reflects a variety of conditions involved in infection with *Bacillus abortus*. Among these conditions are its time relation to the act of abortion and the length of time the abortion bacilli live and multiply in the pregnant uterus. It is obvious that if a uterus be infected in the 8th month of pregnancy, the opportunity for agglutinins to accumulate are poorer than if the uterine infection lasts 3 or 4 months. The presence of *Bacillus abortus* in the udder determines in many cases the intensity of the reaction. No definite rules can therefore be formulated for the interpretation of the agglutination reaction quantitatively, since it is bound up with a complicated process varying from case to case. In the individual cow in general a titer of 1:40 or less may be regarded as indicating that the cow is not infected with *Bacillus abortus* at the time of the blood examination. It does not exclude former infections in the case of older cows, nor does it absolutely exclude very recent infection (Nos. 278 and 351).

[11] The second test of the milk of No. 188 was negative.

The highest titers, 1:640 and above, generally indicate recent infection and in the absence of recent premature births infection of the udder. Even when abortion has just occurred, it may be due to other agencies and the high titer maintained by a chronic infection of the udder dating from an earlier uterine infection with *Bacillus abortus*. Intermediate titers may indicate a gradual rise or decline of agglutinins preceding or following abortion without infection of the udder. They may also stand for a relatively high resistance or partial immunity of the cow.

In any herd a uniformly low titer (1:40 or less) in all animals may be regarded as indicating the entire absence of *Bacillus abortus*. A high titer in any one cow serves to indicate quite definitely the presence of infection in the herd. To determine more accurately the character of the infection in any individual cow there is needed in addition to the quantitative agglutination test a bacteriological study of the milk and of any prematurely discharged calf or fetus.

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